

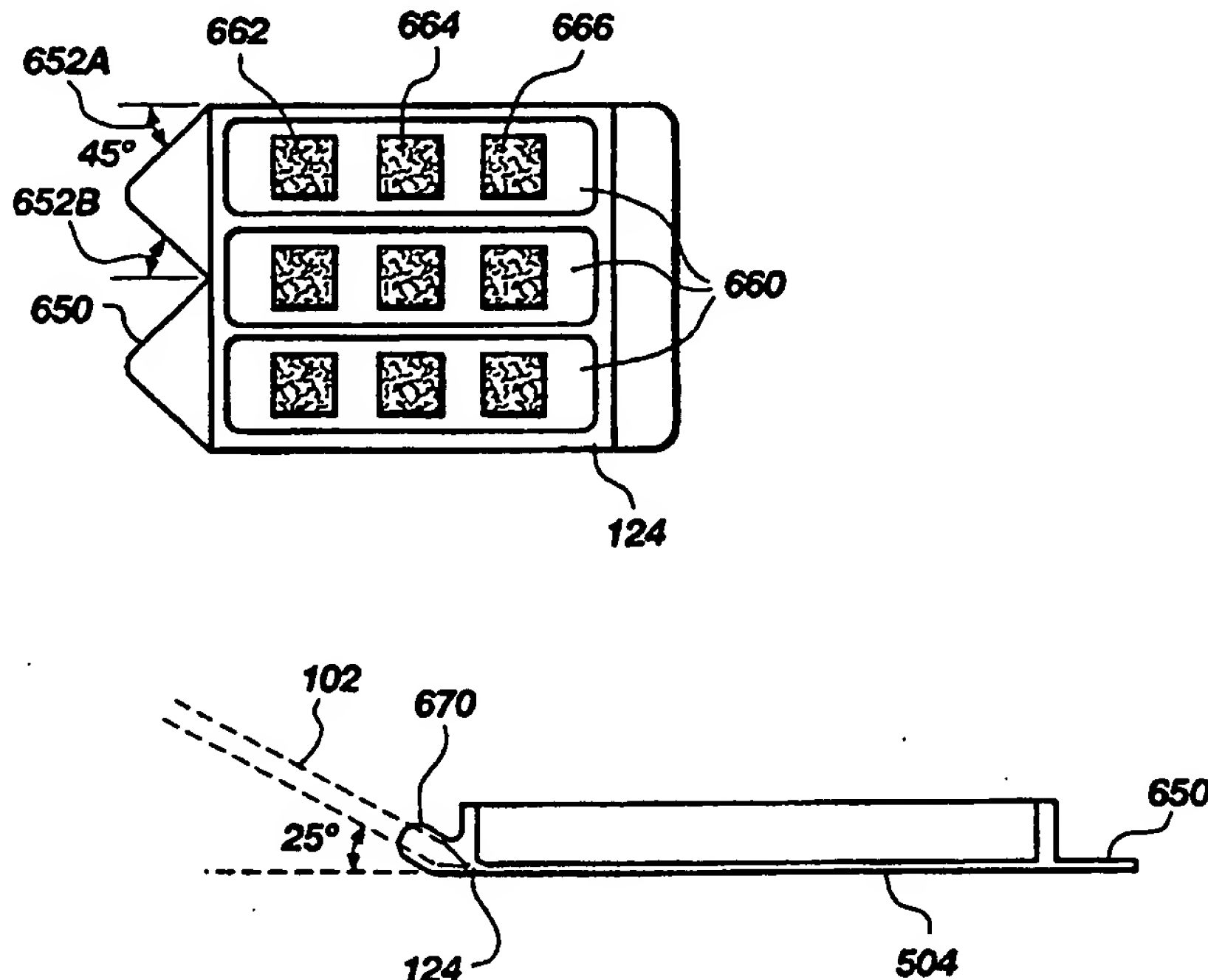
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(54) Title: APPARATUS AND METHODS FOR MULTIANALYTE HOMOGENEOUS FLUOROIMMUNOASSAYS

## (57) Abstract

Methods and apparatus for evanescent light fluoroimmunoassays are disclosed. The apparatus employs a planar waveguide (124) with an integral semi-cylindrical lens (502), and has multi-analyte features and calibration features, along with improved evanescent field intensity. A preferred embodiment of the biosensor (120) and assay method have patches (662) of capture molecules (720) each specific for a different analyte (210), disposed adjacent within a single reservoir (660). The capture molecules (720) are immobilized to the patches (662) on the waveguide surface by site-specific coupling of thiol groups on the capture molecules (720) to photo-affinity crosslinkers (706), which in turn are coupled to the waveguide surface (700) or to a non-specific-binding-resistant coating (702) on the surface (700). The patches (662, 664, 666) of different antibodies are produced by selectively irradiating a portion (714) of the waveguide surface (700) during the process of coupling the photo-affinity crosslinkers (706), the selective irradiation involving a mask (712), a laser light source, or the like.



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# APPARATUS AND METHODS FOR MULTIANALYTE HOMOGENEOUS FLUOROIMMUNOASSAYS

## Background of the Invention

5        Technical Field: This application relates to the art of analyzing samples for particular substances by means of fluorescent binding assays, and more particularly to apparatus, compositions and methods for such assays employing evanescent light.

State of the Art: Biosensor apparatus based on optical detection of analytes by fluorescence of tracer molecules, have attracted increasing attention in recent years.  
10    Such apparatus are useful for both diagnostic and research purposes. In particular, biosensors for a solid-phase fluoroimmunoassay, in which an antibody or antibody fragment specific to the desired analyte is immobilized on a substrate, and binding of the analyte to the antibody results either directly or indirectly (for example, by means of a labelled tracer) in a fluorescence signal, are becoming an important class of  
15    optical biosensor.

      In most solid-phase fluoroimmunoassays, to achieve adequate sensitivity a "wash" step is required to remove unbound tracer before measuring the fluorescence. This problem is particularly true for detection of analytes present at concentrations below nanomolar, as is the case for many analytes of interest in body fluids including  
20    blood, serum and urine. However, the wash step is tedious, and care on the part of the technician is required to produce repeatable and accurate results. Accordingly, it is highly desirable to provide a fluoroimmunoassay system in which sensitivity to analyte concentrations of  $10^{-10}$  to  $10^{-13}$  molar or below is achieved without a wash step.

      An optical technique known as total internal reflection (abbreviated TIR)  
25    provides one approach to such a system. Evanescent light is light produced when a light beam traveling in a waveguide is totally internally reflected at the interface between the waveguide and a surrounding medium having a lower refractive index. A portion of the electromagnetic field of the internally reflected light penetrates into the surrounding medium and constitutes the evanescent light field. The intensity of  
30    evanescent light drops off exponentially with distance from the waveguide surface. In a fluoroimmunoassay, evanescent light can be used to selectively excite tracer molecules directly or indirectly bound to an immobilized binding agent, while tracer molecules free in solution beyond the evanescent penetration distance are not excited and thus do not contribute "background" fluorescence. The use of evanescent field  
35    properties for fluorescence measurements is sometimes referred to as evanescent sensing. For a glass or a similar silica-based material, or an optical plastic such as

polystyrene, with the surrounding medium being an aqueous solution; the region of effective excitation by evanescent light generally extends about 1000 to 2000 Å (angstroms) from the waveguide surface. This depth is sufficient to excite most of the tracer molecules bound to the capture molecules (antibodies, receptor molecules, and the like, or fragments thereof) on the waveguide surface, without exciting the bulk of the tracer molecules that remain free in solution. The fluorescence thus resulting reflects the amount of tracer bound to the immobilized capture molecules, and in turn the amount of analyte present.

The tracer fluorescent light will conversely also evanescently penetrate back into the waveguide and be propagated therein. The maximum solution depth for efficient evanescent collection by the waveguide approximates the depth of the region of evanescent penetration into the solution, and thus the waveguide-penetrating portion of the tracer fluorescence can also be used to selectively measure fluorescence from tracer bound to the waveguide surface.

U.S. Patents Nos. RE 33,064 to Carter, 5,081,012 to Flanagan et al, 4,880,752 to Keck, 5,166,515 to Attridge, and 5,156,976 to Slovacek and Love, and EP publications Nos. O 517 516 and O 519 623, both by Slovacek et al, all disclose apparatus for fluoroimmunoassays utilizing evanescent sensing principles.

Desirably, an immunofluorescent biosensor should be capable of detecting analyte molecules at concentrations of  $10^{-12}$  M (molar) or below. To date, most reports of evanescent-type biosensors indicate that at best, concentrations of  $10^{-11}$  M could be detected.

It is further desirable for speed and convenience in "routine" testing, for example testing of blood bank samples for viral antibodies, to have an evanescent immunofluorescent biosensor which is disposable and which provides multi-sample measurement capability. Multi-sample capability would allow a test sample and a control sample (such as a blank, a positive control, or for a competition-type assay, a sample preloaded with tracer molecules) to be simultaneously illuminated and measured. Simultaneous multi-sample capability would also speed up the process of analyzing multiple samples and would reduce the effects of variation in the level of exciting light which are known to occur with typical light sources. However, in a typical prior art evanescent light device such as that of Block et al, U.S. Patent No. 4,909,990 issued March 20, 1990, the waveguide is a fiber optic rod whose shape makes it difficult to build a multi-well biosensor.

Another factor which affects the attainable sensitivity relates to the intensity of excitation light emitted from the waveguide. The intensity of fluorescence emitted by tracer molecules is in part dependent on the intensity of exciting light (which is the evanescent field). Therefore, increased evanescent light intensity should provide  
5 increased fluorescence which in turn would improve the detection sensitivity. The level of evanescent light is in turn dependent on the intensity of the light beam propagating in the waveguide, and this can be increased by decreasing the cross-sectional area of the waveguide.

Previous methods of immobilizing antibodies to optical substrates in evanescent  
10 biosensors also present some problems causing reduction in sensitivity. Many such methods utilize the  $\epsilon$ -amino groups of lysine residues in the protein. This approach has at least two significant disadvantages due to the fact that most proteins have multiple lysine residues. First, the presence of multiple potential coupling sites (multiple lysine residues) results in multiple random orientations of antibodies on the  
15 substrate surface. If the substrate-coupled lysine residue is near the N-terminal of the antibody molecule, the antibody's antigen binding site (which is near the N-terminal) may be effectively unavailable for binding of the analyte.

Second, if multiple lysines on the same antibody molecule are coupled to the substrate, the molecule may be subjected to conformational strains which distort the  
20 antigen binding site and alter its binding efficiency. For capture molecules immobilized by typical prior methods, generally only 20% or less of the binding sites are functional for analyte binding. Thus, it is desirable to have a site-specific method for coupling of the antibodies or other proteins, so that the capture molecules will be uniformly oriented and available for analyte binding.

25 Another problem relates to the levels of non-specific binding to the antibody-coated surface of the optical substrate. These levels are often sufficiently high to make detection of analyte at concentrations below about  $10^{-10}$  M very difficult. Nonspecific binding can be reduced by including a wash step after the sample is incubated with the coated substrate, to remove unbound tracer molecules. However,  
30 as discussed above, a wash step is undesirable. Second, non-specific binding can be a serious problem unless the surface is "passivated" with a masking agent such as bovine serum albumin or with a thin coating of hydrophilic polymer such as poly(ethylene glycol) or poly(methacrylate). Without such passivation (which introduces yet another step into the procedure), non-specific binding can be 50% or



more of the specific binding. Even with passivated surfaces, non-specific binding can be sufficient to reduce detection sensitivity and reproducibility.

Thus, a need remains for an evanescent biosensor system which provides the desired sensitivity in a homogeneous assay (homogeneous being defined for purposes  
5 of this application as meaning an assay that does not require a wash step). A need further remains for such an apparatus with improved sensitivity for detection of analytes at picomolar concentrations and below. A need also remains for an immunofluorescent assay and biosensor with properties of low non-specific binding and having uniformly oriented capture molecules. A need also remains for such a  
10 biosensor and assay system which are inexpensive and readily used by non-skilled persons.

### Summary of the Invention

The invention comprises a system including both apparatus and methods for a  
15 homogeneous immunofluorescence assay based on evanescent light principles, capable of detecting one or more analytes at concentrations less than pico-molar. The overall configuration of the apparatus is such that fluorescence-emitting tracer molecules bound to a waveguide surface are excited by an evanescent field penetrating into the adjacent solution from a light beam propagated within the waveguide, the propagated  
20 beam being introduced at an end or edge of the waveguide. The emitted fluorescence is then directly collected from the zone of evanescent penetration, e.g. not from an edge or end of the waveguide.

The apparatus includes a biosensor comprising a planar waveguide having first and second parallel plane surfaces and an edge extending between them, the edge  
25 having a receiving region for receiving light to be internally propagated. A semi-cylindrical lens is integrally adapted to the waveguide adjacent the receiving region, at least one of the waveguide surfaces has a plurality of capture molecules immobilized thereon. The capture molecules are configured to specifically bind a chosen analyte; the capture molecules may be antibodies, antibody fragments, haptens, membrane  
30 receptors, or any useful specific binding agent. The capture molecules may include a plurality of species each specific for a different analyte, and different species may be localized in different and mutually exclusive regions on the waveguide surface.

The apparatus further includes a light source configured and disposed to deliver a sheet beam of light into the waveguide through a receiving region on the

edge, and detection means disposed for direct collection of the fluorescence from the evanescent zone, direct collection being defined as not requiring penetration of the fluorescence into the waveguide. The detection means is desirably an imaging detector configured to simultaneously separately collect a plurality of fluorescence signals each originating from a different region on the waveguide surface. The imaging detector includes a plurality of photodetection elements spaced from each other in the displaced parallel plane, and lens means positioned to focus each of said fluorescence signals onto a respective one of the photodetection elements.

In a highly preferred embodiment, the semi-cylindrical lens and the waveguide are integrally molded of an optical plastic, and the lens is oriented to aim the beam at a selected angle to the plane of the waveguide, the selected angle being less than the critical angle of reflection at the waveguide-liquid interface. The waveguide also may have a serrated portion forming the portion of the edge opposite the receiving region.

In other preferred embodiments of the biosensor, the waveguide surface is an optical substrate coated with a passivating coupling coating which reduces non-specific binding. The capture molecules may be linked to the coupling coating via a photo-affinity crosslinking agent. The capture molecules may also be coupled to the coating in a site-specific manner.

The invention further encompasses methods for immobilizing the capture molecules to the waveguide surface, of producing the optical substrates with patches of different capture species, and methods for performing an evanescent fluorescent immunoassay without a wash step. A method for producing the waveguide with patches of different capture molecules involves the use of photo-affinity crosslinking agents in combination with a masking system to selectively crosslink capture molecules to selected regions of the waveguide surface. In a highly preferred method, the waveguide surface is coated with a coating agent which inhibits non-specific protein binding, and the photo-activated crosslinking agent couples the capture molecules to the coating via a thiol site on the capture molecules. The latter coupling provides site-specific immobilization of the capture molecules, so that 50% to 70% of the capture molecules have the analyte binding sites readily available for binding. The method also provides for non-specific binding levels of less than 5% to 10% and as low as 1-2%.

Brief Description of the Drawings

FIG. 1 is a schematic diagram of a fluorescent immunoassay apparatus of the invention;

FIG. 2 is a side view of a portion of the waveguide and the biochemical  
5 components of a competition immunofluorescent assay according to the invention;

FIG. 3A is a top view of a flow biosensor of the apparatus of FIG. 1;

FIG. 3B is a side cross-section view of the flow biosensor taken along line B-B  
in FIG. 3A;

FIG. 3C shows the waveguide in isolation as it could be arranged with respect  
10 to a cylindrical lens and incoming and reflected light waves;

FIG. 4A is an elevational view of a two-channel flow biosensor of FIGS. 3A-  
3B with respect to exciting light beams and the collection of fluorescence in an  
immunoassay;

FIG. 4B is a schematic diagram of the two-channel biosensor indicating the  
15 arrangement of two components of the detection device, a CCD detector and the  
entrance spectrometer slit, with respect to the waveguide regions;

FIG. 4C is a diagram of fluorescence intensities as they might be detected  
from the two channels of a biosensor arranged according to FIGS. 4A and 4B;

FIG. 5A is an elevational view of an alternate embodiment of a multi-channel  
20 biosensor;

FIG. 5B is a side view of the biosensor of FIG. 5A;

FIG. 5C is a side view of the biosensor of FIG. 5A in a vertical orientation  
with a sample solution therein;

FIG. 5D is a cross-sectional view of the biosensor taken along line D-D in  
25 FIG. 5C;

FIG. 6 is an elevational view of an alternate embodiment of a multiwell  
biosensor;

FIG. 7A is a chart depicting fluorescence intensity data from a sandwich  
fluoroimmunoassay for detecting an antibody, and performed with the apparatus of  
30 FIG. 1 according to a first assay format;

FIG. 7B is a chart depicting data from a sandwich fluoroimmunoassay  
performed with the apparatus of FIG. 1 according to an alternate assay format;

FIG. 8 is a chart comparing the fluorescence enhancement observed with the  
assay formats of FIGS. 7A and 7B;



FIGS. 9A-F are charts depicting data from an alternate scheme for a sandwich fluoroimmunoassay for detecting an analyte using a corresponding antibody;

FIGS. 10A-D are charts depicting data from a displacement fluoroimmunoassay performed with the apparatus.

5 FIG. 11A is an elevational view of another embodiment of a biosensor;

FIG. 11B is a side cross-section view of the biosensor embodiment of FIG. 11A;

FIG. 11C is an end view of the biosensor embodiment of FIG. 11A;

10 FIGS. 12A and 12B are a top view and an elevation view, respectively, of an alternate embodiment of an integrally molded biosensor;

FIG. 13 is a side view of a molded biosensor with an alternate embodiment of the integral lens;

FIGS. 14A and 14B are a side view diagram of an improved imaging photo-detection system and a top view of a photodiode array for use in the system;

15 FIG. 15 is a perspective diagram of a photo-masking set-up for producing a waveguide with patches of different Fab' species;

FIG. 16 is a side view of a section of the waveguide surface showing in schematic form, steps in a process of patterning a waveguide surface with different Fab' species;

20 FIG. 17 is a side view of a waveguide surface showing an alternate embodiment of the patterning process;

FIGS. 18A, 18B, 18C and 18D show chemical formulas of photo-affinity crosslinkers useful in the invention, and partial chemical reactions for photo-coupling the crosslinkers to a base coating.

25

#### Detailed Description of the Illustrated Embodiments

A light source 100 provides a light beam 102 which is directed by means of mirrors 104, 106, 108 to an optical biosensor indicated generally at 120 (FIG. 1). In the working embodiment, light source 100 is an argon laser capable of emitting light  
30 at wavelengths of between about 488 and 514.5 nanometers (abbreviated nm). In an alternate embodiment, a laser diode emitting at wavelengths of 600 to about 700 nm can be used as light source 100. Depending on the requirements of the fluorescent tracer, light source 100 may also be embodied as any other laser or other high-

intensity light source emitting a sufficient amount of light at an appropriate wavelength to excite the selected tracer.

The embodiment of FIG. 1 further includes a 45° angle mirror 110 which is positioned for making beam 102 a vertical beam prior to focussing the beam onto the biosensor. It will be understood by those skilled that the number and arrangement of mirrors 104, 106, 108, 110 may be varied as necessary to accommodate various space limitations, with the sole requirement being that a sufficient amount of light be directed to biosensor 120.

Biosensor 120 has an optical substrate 122 with one end 124 positioned to receive light from beam 102. A focussing lens 126 is positioned between angle mirror 110 and end 124 of waveguide 122, for focussing light from beam 102 onto end 124. Focussing lens 126 is here shown mounted on an X-Y translation unit so that its position may be adjusted for best focussing. In contrast to the rod-shaped fiber optic waveguides typically found in immunofluorescent assay devices, in the present invention optical substrate 122 is of generally planar shape having two planar surfaces spaced by a width, as shown in FIG. 2. Optical substrate 122 may for example be a square or rectangular glass microscope slide or coverslip, or the like. Materials for optical substrate 122 include glass, high-lead glass, quartz, optical plastic, and the like as are well-known in the art.

Light detection means indicated generally at 150 are positioned to detect fluorescent light emitted from biosensor 120. The emitted light is reflective of the concentration of a selected analyte in a sample, as is better described subsequently in reference to FIGS. 2 and 7-10. Light detection means 150 includes a collection lens 152 positioned to collect the emitted fluorescence from a direction substantially orthogonal to the direct of propagation of light beam 102 through optical substrate 122.

The distance 154 between collection lens 152 and optical substrate 122 is selected as known to those skilled to maximize the collection of light emitted from the region of evanescent light penetration. The light collected by collection lens 152 is then sent to detection means 150, which responds by outputting signals reflective of the level of collected fluorescence light.

Detection means 150 may be any type of photodetector useful to detect light in the wavelength region spanning the wavelength range of the emitted fluorescence, as known in the art. However, in a preferred embodiment for simultaneous multi-analyte

assays, detection means 150 is an imaging-type detector providing direct imaging of each of the fluorescent signal(s) originating in the evanescent zone 240. In the apparatus of FIG. 1, detection means 150 is a CCD (charge-coupled device) detector which produces a signal like that depicted in FIG. 4C. Such imaging signal collection provides simultaneous measurement of multiple samples in a much simpler way than a system in which a separate optical element is needed to read each well or patch. The present imaging detection system also provides for collection of emitted fluorescence directly from the evanescent zone 240 (FIG. 2), rather than via evanescent penetration of the fluorescence into the waveguide.

Alternatively, detection means 150 may be a photomultiplier, a semiconductor photodiode, or an array of such detectors. In embodiments other than a CCD, an array is generally preferable to a single detector for some purposes. With an array of small detectors, the user can determine that the peak fluorescence is being detected and is not inadvertently missed due to misalignment of the collection and detection optics. Optionally, a grating spectrograph is coupled to the CCD or other detection means, to provide spectral analysis of the detected light. In that case, means are also provided to integrate the signal function around each peak to determine the total collected fluorescence from a sample. Alternatively, in an embodiment for use in a setting such as in a testing laboratory, and for which all the parameters of the assay have been standardized, the spectrograph may be replaced by a filter which passes only wavelengths in the region of tracer fluorescence.

FIGS. 14A and 14B depict an alternate and presently preferred embodiment of an imaging detection system, which may be substituted for the CCD imaging detector of FIG. 1. In this embodiment, an array of photodiodes 680 is arranged with respect to a detection lens means such that light from a given patch is focussed onto a corresponding photodiode (FIG. 14A). In this embodiment, the detection lens means comprises a pair of opposingly oriented lenses 682, 684. The apparatus having the imaging detection system of FIGS. 14A-B is substantially less expensive to make than one having a CCD detector. The patches 662, 664, 666 should be spaced appropriately to correspond to the size and spacing of the photodiodes, the focal length and magnification of the lens, and the distance between the waveguide surface, the detection lens or lenses, and the photodiode array, as generally understood in the art of optics.

Desirably, one or more filters 688 are positioned adjacent the detection lens, and preferably between two detection lenses as shown in FIG. 14B. This arrangement provides for effective filtering of scattered excitation light and other stray light prior to impingement of the signal light on the photodiodes. The filter(s) can be of either  
5 bandpass or long-pass type, and the wavelength region to be passed will depend upon the wavelengths of the excitation light and the fluorescent light of the tracer molecule. For example, with laser diode excitation at 635 nm of the cyanine dye CY5 which has peak fluorescence at 670 nm, filters passing light longer than about 650 nm in wavelength are useful. In a presently preferred embodiment, a bandpass filter  
10 centered at 670 nm with a 40 nm line-width is used.

For focussing light beam 102 onto the end of the planar substrate waveguide, it is preferred to replace the typical spherical lens with a lens of semi-cylindrical shape, as better seen in FIGS. 3C, 5A, and 11A. For purposes of this application, "semi-cylindrical" is defined to include both a transection of a right circular cylinder along a  
15 plane parallel to the vertical axis of the cylinder, and a transection of a right cylinder having bases of an elliptical shape. Thus, the lens shape may be similar to the type known as aspherical. A hyperboloid cross-section may also be suitable. The shape and dimensions of the curved surface of the lens should however be longitudinally uniform along the region used for focussing of the light beam.

20 As is better seen in FIG. 2, optical substrate 124 is embodied as a planar waveguide having at least one planar surface 200 spaced from a second surface 201 by a width 202. At least surface 201 is disposed in contact with a sample solution 203. A plurality of capture molecules 204 are immobilized on surface 201. The sample solution contains a plurality of analyte molecules 210 of a selected analyte, and a  
25 plurality of tracer molecules 220. The capture molecules are chosen or constructed to bind to a binding moiety present on each of the analyte molecules 210. The tracer molecule 220 is chosen or constructed to emit fluorescent light in response to stimulation by light of the appropriate wavelength. The level of fluorescence emitted by the tracer molecules 220 is a measure of the amount of analyte bound to the  
30 capture molecule and is thereby reflective of the concentration of analyte molecules 210 in the solution.

When light is being propagated in the waveguide 124 and internally reflected at the surfaces 200, 201, an evanescent light field is produced having an intensity curve 230 which drops off with distance from the surface 200, as diagrammed relative to a

distance axis 232 and an intensity axis 234 (not to scale). An excitation zone 240 is the only region of the solution in which the evanescent light intensity is sufficient to excite a significant or detectable fraction of tracer molecules 220 (not to scale). Tracer molecules 220 outside zone 240 will contribute little or no induced  
5 fluorescence. Excitation zone 240 is typically between about 1000 and 2000 Å in depth.

Capture molecules 204 may be whole antibodies, antibody fragments such as Fab' fragments, whole antigenic molecules (haptens) or antigenic fragments, and oligopeptides which are antigenic and/or similar in 3-dimensional conformation to an  
10 antibody-binding epitope. Capture molecules 204 may also be a receptor molecule of the kind usually found on a cell or organelle membrane and which has specificity for a desired analyte, or a portion thereof carrying the analyte-specific-binding property of the receptor.

In FIG. 2, a competition assay scheme is depicted (also termed a displacement  
15 assay). However, as will be apparent to the skilled person, alternate assay schemes such as sandwich assays may be performed with the present apparatus.

The capture molecules 204 may be immobilized on the surface 202 by any method known in the art. However, in the preferred embodiment the capture molecules are immobilized in a site-specific manner. As used in this application, the  
20 term "site-specific" means that specific sites on the capture molecules are involved in the coupling to the waveguide, rather than random sites as with typical prior art methods. Examples I-III detail methods for site-specific immobilization of capture molecules to the surface of the optical substrate by means of a protein-resistant coating on the substrate.

25 As previously stated, the intensity of evanescent light drops off rapidly with distance from the waveguide surface. Thus, only tracer molecules which are within an effective excitation range 240 (not necessarily to scale) from the waveguide surface, will be excited by the evanescent light to emit fluorescence. The range 240 is generally about 1000 to 2000 Å. This range is sufficient to ensure that essentially  
30 all tracer molecules 220 which are bound (directly or indirectly) to capture molecules 204, will be detected, while the bulk of the tracer molecules which remain free in solution are outside the effective excitation range.

In a working embodiment of the apparatus of FIG. 1, measurements of fluorescence are made by spectroscopy. For the examples involving rhodamine-



tagged molecules, light source 100 is an argon ion laser (a LEXEL Model 95-2) at an emission wavelength of 514 nm. Fluorescence detection was done with a monochromator (SPEX Industries, Inc., Model 1680C) and a charge-coupled device (abbreviated CCD) (Photometrics Ltd. Series 200, or CH-250). Alternatively, light source 100 can be any light source emitting at the wavelength desired for excitation of selected fluorescent dyes. Also, once an assay procedure has been validated and standardized, it may not be necessary to measure the fluorescence spectrum or spatial distribution of fluorescence. The detection means may be simplified in accordance with the minimum requirements of the assay.

10 In another alternate embodiment, light source 100 is a laser diode emitting in the red wavelength region of 600-700 nm, available from Toshiba (model No. TOLD 9211) This laser diode provides about 5 milliwatts of power with a peak emission wavelength of about 670 nm. Laser diodes emitting at 630 nm are also available and can be used. For an embodiment using a wavelengths in this region, it is necessary to use dyes such as cyanine dyes, whose fluorescence can be stimulated by excitation with wavelengths in the red spectral region. An example of such a dye is CY5, available from Biological Detection Systems, Inc., Pittsburgh PA (catalog no. A25000). The CY5 dye can be conjugated to the desired tracer molecule by the manufacturer's instructions and/or with a kit available from BDS. A second dye, 20 CY7, which is available from the same source may also be suitable. The dyes and methods for conjugating are also characterized in the paper by Southwick, P.L., et al., titled "Cyanine Dye Labelling Reagents - Carboxymethylindo-cyanine Succinimidyl Esters", Cytometry 11:418-430 (1990). The use of laser diodes as a light source permits the biosensor and waveguide to be formed of plastic, which 25 considerably reduces the expense of manufacture and facilitates the integral molding of the semi-cylindrical lens with the waveguide and reservoirs.

In the embodiment of FIG. 2, the immunoassay is a competition assay in which the tracer molecules 220 are constructed such that capture molecules 204 will bind tracer molecules 220 in place of analyte molecules 210. Higher concentrations of analyte molecules 210 will cause most of the tracer molecules 220 to be displaced 30 into the surrounding solution from capture molecules 204, thus reducing the number of tracer molecules within excitation range 240 of the substrate 122. This reduced binding of tracer molecules in turn reduces the amount of fluorescence. In contrast,

lower concentrations of analyte molecules 210 will allow tracer molecules 220 to bind to capture molecules 204, and thus to be held within the excitation range 240.

In the embodiment of FIG. 1, biosensor 120 is shown as a flow-through cell, shown in greater detail in FIGS. 3A-B. A planar waveguide 302 which may be for example a microscope slide or coverslip, is sandwiched between two plates 304, 306 which are held together by screw fittings 308A, 308B. A gasket 320 is seated between waveguide 302 and plate 306. Gasket 320 is configured with two internal openings which, when gasket 320 is securely sandwiched between plate 306 and waveguide 302, form reservoirs 322, 324. In reservoirs 322, 324, waveguide 302 constitutes one wall, plate 306 constitutes a second wall, and the inner edges 322A, 324A of the gasket form the remaining walls. Although the reservoirs 322, 324 are here shown to be rectangular in shape, other shapes could be used. Also, instead of two reservoirs as depicted in FIG. 3A, the gasket could have either just one opening or more than two, creating corresponding numbers of individual reservoirs.

Gasket 320 is preferably made of a semi-rigid material having an index of refraction less than that of the waveguide material in the wavelength range of the exciting light. For best results, it is believed that the index of refraction of the gasket material should be as low as possible compared to that of the waveguide. For a waveguide made of quartz or glass the index of refraction would typically be from about 1.46 to 1.52, higher for high-lead glass. A transparent (non-pigmented) silicon rubber (siloxane polymer) with an index of refraction of 1.35-1.43 is a presently preferred material for gasket 320. TEFLON-type materials such as PTFE (polytetrafluoroethylene) or FEP (fluorinated ethylene propylene) have indices of refraction of around 1.34-1.35, and may also be suitable. However, because TEFLON surfaces tend to adsorb protein in a non-specific manner, silicon rubber is generally preferred.

The lower plate 306 in FIG. 3B, has a pair of inlets 330, 332 and a pair of outlets 340, 342. These inlets and outlets are arranged so as to permit solutions to flow separately through the respective reservoirs 322, 324. Desirably, the lower plate 306 may be made from aluminum alloy.

FIG. 3C shows the waveguide 302 in isolation from the remaining parts of the biosensor. Lens 126 is shown receiving and focussing light beam 102 onto the waveguide. Desirably, the outer, surrounding edge 350 is coated with a reflective material, except for an uncoated region 352 at which the focussed light from lens 126

enters the waveguide (FIG. 3C). Arrows 354 indicate reflection from the coated edges. In FIG. 3C, only one lens and one uncoated region are shown, however, for two or more channels, more portions of edge 350 may be left uncoated to allow light to enter the waveguide (see for example FIG. 4A).

5       The reflective coating reflects back into the waveguide, light that would otherwise escape through the edge 350. The intensity of the evanescent light wave is thereby enhanced. Suitable reflective coating materials include aluminum, silver, or the like, as known in the art. Alternatively, in place of a coating, reflectors could be positioned about the edges to reflect escaping light back into the waveguide.

10       The design with at least two individual reservoirs has significant advantages over a single reservoir embodiment for instances in which it is desirable to measure the test sample fluorescence simultaneously with fluorescence from a control region on the same waveguide. For example, the level of non-specific binding to the waveguide can be subtracted from the test sample fluorescence. Also, measurement changes due  
15 to fluctuations in intensity of the exciting light can be corrected for. In a displacement assay, the "control" region could be the pre-loaded waveguide with no analyte present in the sample, or with a known amount of analyte. With three or more wells, fluorescence can be measured for both a no-analyte control and at least one known, calibration analyte sample in addition to the "unknown" or test sample.

20       Fig. 4A depicts the flow-through cell of FIGS. 3A-3B as it would be used for a waveguide excitation protocol. Here, the light beam is split into two equal components 400, 402 passing through respective focussing lenses 404, 406 to illuminate "channel 1" (CH.1) and "channel 2" (CH.2) in the waveguide 302. Solid  
25 arrows 410 indicate the direction from which fluorescence in CH.1 is collected, while dashed arrows 412 indicate the direction from which fluorescence in CH.2 is collected.

When the focussing lenses 404, 406 are properly aligned with respect to waveguide 302 and the light source, two illuminated strips 430, 432 (FIG. 4B) are visible which extend down the waveguide in the direction in which beam components  
30 400, 402 are aligned. Box 440 represents an approximate outline of the detection region of the CCD array while box 442 represents an approximate outline of the spectrograph entrance slit. As previously described, one embodiment of a detection system comprises a spectrograph in combination with a CCD array. FIG. 4C depicts hypothetical expected results from such a detection system for simultaneous

measurement of a "blank" or no-analyte sample in CH.1 and a test sample or "unknown" in CH.2. Curves 450, 452 respectively represent the fluorescence from CH.1 and CH.2. The fluorescent intensities of the blank and the sample would be compared by means of the values of the corresponding integrals of curves 450, 452 over the respective regions 460, 462. A calibration curve for a series of calibration samples of known analyte concentrations would typically be made and used to determine the concentration of an unknown sample, as known in the art.

Of further interest in FIG. 4A is the orientation of lenses 404, 406 with respect to waveguide 302. It will be seen that the curved edges 404A, 406A face towards waveguide 302, which is 180° from the orientation depicted in FIG. 3C. While the focussing lens can be oriented in either way, the arrangement of FIG. 4A is presently preferred for illumination of the waveguide with the flow-through cell.

FIGS. 5A-5D depict an alternate embodiment of a biosensor useful with the apparatus of FIG. 1. The biosensor indicated generally at 500 has an integrally mounted or formed focussing lens 502 and waveguide 504 arranged such that lens 502 focusses light onto the forward end 506 of the waveguide. Focussing lens 502 is configured and positioned to focus a light beam onto the receiving end 506 of the waveguide 504 (FIGS. 5A, 5C). Side walls 511, 512, top and bottom walls 516, 517, and a removably sealing rear wall 518 enclose the space about the waveguide 504 to create reservoirs 520, 522.

The integral focussing lens 502 replaces focussing lens 126 in the apparatus of FIG. 1. In the working embodiment of FIGS. 5A-5D, the focussing lens is molded as part of the waveguide holder 500 of an optical plastic such as polystyrene, polycarbonate or the like.

Biosensor 500 also includes reservoirs 520, 522 best seen in FIGS. 5B, 5C and 5D in which sample solutions can be disposed. Optionally, for some applications it may be desirable to provide lengthwise ribs 530 (FIG. 5D) along slot 504 which can define separate regions of the waveguide surface.

FIG. 6 depicts an alternate multiwell biosensor similar to that of FIGS. 5A-5C, except that a series of discrete wells 600, 602, 604, 606 are formed on the waveguide 504. The embodiment of FIG. 6 would be used in a horizontal position, so that the wells 600, 602, 604, 606 need not be covered.

The biosensor including the lens may be formed by molding of a suitable optical plastic. A holder comprising the reservoir walls, the lens, and frame elements

as needed, may be pre-molded. A silica-surface waveguide is inserted subsequently with a refractive-index-matched adhesive to secure it in place and seal it as needed to create separate channels. Alternatively, the holder may be molded with a silica-surface waveguide in place, thereby eliminating the need for the adhesive.

5 In a presently preferred further embodiment, the waveguide is also formed of the optical plastic and is molded simultaneously with the lens and/or the reservoirs. The latter type construction is not suitable for use with excitation wavelengths of 488 to 515 nm, because known optical plastics tend to emit fluorescence when excited in this (the blue and green) wavelength region. This fluorescence would appear as  
10 background fluorescence. However, an alternate embodiment of the apparatus using a light source emitting at wavelengths of 600 nm and above, would accommodate a plastic waveguide. Molding the lens/wave-guide, or lens/waveguide/reservoir(s), as a single unit of plastic substantially reduces the cost of manufacturing and makes a disposable biosensor more feasible.

15 FIGS. 11A-C shows another embodiment of a biosensor similar to that depicted in FIGS. 3A-C. In FIG. 11A, waveguide 10 is a glass coverslip inserted in a sawcut groove 12 (FIG. 11B) in a solid, colorless plastic lens 14. Transparent walls 16, 18, 20, 22 are sealingly attached with index-matched adhesive to the lens 14 and about the edges of the waveguide 10 to form a pair of separate reservoirs 30, 32  
20 (FIG. 11B).

In the embodiment of FIGS. 11A-C, the curved forward edge 34 of the lens 14 is spaced at a distance 40 from the forward end of the waveguide 10. Distance 40 is selected so as to match the focal length of the lens 14. A mask 42 made of a material that is opaque to visible light, covers the rear edge 44 of lens 14. The embodiment of  
25 FIGS. 11A-C can be used in a vertical orientation as shown in FIG. 5C. Alternatively, the biosensor may be oriented with the waveguide 10 in a substantially horizontal position, so that only one side of the waveguide 10 is used. In such case, a cap which can sealingly close the open ends of the reservoirs must also be provided. An advantage of the horizontal orientation scheme is that only a thin layer 50 of  
30 sample solution is required (FIG. 11C). However, unless ribs along the waveguide 10 are provided, like ribs 530 in FIG. 5D, the biosensor of FIG. 11C in the horizontal orientation has only one effective sample channel.



While the curved edge 34 of lens 14 is shown as being substantially a semi right-cylinder in shape, other lens shapes are possible as described previously herein with respect to FIGS. 3A and 5C.

In a further and highly preferred embodiment of the biosensor depicted in  
5 FIGS. 12A and 12B, the end of the planar waveguide which is distal to the receiving edge, has a portion 650 of serrated or toothed shape. The angles 652A, 652B must be less than the critical angle for total internal reflection at the waveguide-air interface (the critical angle for polystyrene/air is about  $51^\circ$ ). Preferably, the sum of angles 652A, 652B should be  $90^\circ$ , so that the light is retro-reflected directly back along the  
10 longitudinal axis of the waveguide; still more preferably, angle  $652A = 652B = 45^\circ$ . An advantage of this shape is that it increases the level of internal reflection without a reflective coating on the edges, thus reducing manufacturing complexity and costs. The increased TIR enhances the evanescent field intensity and thus improves sensitivity of the assay. Also, the serrated end edge helps to equalize (make more  
15 uniform across the whole waveguide) the intensity of light within the waveguide. The entire waveguide portion of the biosensor, should have an optical-quality surface, including the serrated end and the integral lens.

In a working embodiment, the waveguide is about 0.05 centimeters (cm) in thickness and the wells are about 0.08 to 0.1 cm in depth. The biosensor including  
20 the waveguide is about 2.5 cm wide and about 4.3 cm long.

The embodiment of FIGS. 12A and 12B also has a plurality of parallel wells 660 each extending along the longitudinal direction from the light receiving end 124 of the waveguide. Notably, each well contains a plurality of patches 662, 664, 666 each comprising a different immobilized Fab' species. The elimination of separation  
25 walls between such different species, which would extend crosswise to the direction of light propagation in the waveguide, further increases the sensitivity of the assay. The increased sensitivity results from 1) avoiding leakage of waveguide light through the walls, and 2) avoiding scattering of the excitation light which may excite unbound tracer molecules outside the region of evanescent penetration, undesirably increasing  
30 background fluorescence.

In another improvement, the sheet excitation beam is arranged to enter the receiving edge of the waveguide at an angle to the plane of the waveguide. FIG. 13 shows an angled integral lens 670 configured to accept such angled beam entry. For this purpose, the beam originating from the laser should be shaped to a sheet of width

approximating the width of the receiving region of the waveguide, and of relatively narrow thickness (preferably no more than tenfold, and preferably one- to four-fold the waveguide thickness), using cylindrical and/or spherical lenses as known in the art.

5           The effect of so angling the beam entry is to increase the proportion of light entering the waveguide which excites the higher order modes that are propagated in the multimode waveguide, thereby increasing the intensity of the evanescent field. Desirably, the mean beam entry angle is selected to be less than, but near the critical angle of the waveguide/solution interface. The closer the beam entry angle is to this  
10 critical angle, the greater the increase in evanescent intensity. However, a beam entry angle too near the critical angle could result in some of the entering light escaping at the waveguide/solution interface, thus decreasing the waveguiding efficiency and evanescent intensity. Thus, there is an optimum angle that can be determined experimentally. Generally, a beam entry angle of a few degrees less than the critical  
15 angle (from about 5 to about 15 degrees less), will be useful.

For a polystyrene waveguide, the critical angle is about  $33^\circ$ , and the presently preferred beam entry angle is  $25^\circ$ ; with these values, an increase in evanescent light intensity of at least about a factor of two is achieved over using a  $0^\circ$  beam entry angle.

20           The use of an angled beam entry necessitates adjustment of the orientation of the center of radius of curvature of the semi-cylindrical lens with respect to the waveguide receiving end, as will be evident to a skilled person. In a molded integral biosensor, the integral lens/waveguide may be formed as shown in FIG. 13.

          The following examples detail several methods for attaching the capture  
25 molecules to the waveguide surface in a site-specific manner. The general scheme for reducing the level of non-specific binding is to coat the waveguide with a protein-resistant material, and then immobilize the antibody to the coating. The scheme further includes derivatizing of the protein-resistant coating combined with site-specific modification of the antibody or other capture molecule to be immobilized, so  
30 as to provide site-specific attachment of the capture molecule to the coating.

Of the examples presented, the procedures of Examples I and II gave generally better results. At present, the avidin-biotin coupling method (Example II) is the most preferred. Using either coupling scheme, at least about 75% of the immobilized Fab' fragments were active, and the levels of non-specific binding were typically no more

than 1-2 % of the specific binding. The modified PEG coating gave slightly higher levels of non-specific binding, in the range of 5 % to about 25 %.

### EXAMPLE I

#### 5 PREPARATION OF WAVEGUIDE SURFACE - HYDROGEL

A silica surface was prepared with a hydrogel coating comprised of polymethacryloyl hydrazide (abbreviated PMahy). Fused silica slides of CO grade and thickness about 1 mm, available from ESCO, Inc., were suitable as waveguides (optical substrates).

10 To graft the PMahy to the silica, the surface was derivatized with aldehyde groups. The derivatization was accomplished by silanization with 3-aminopropyltriethoxy silane (abbreviated APS) to add an amino functional group, followed by reaction with glutaraldehyde to produce free aldehyde groups. The PMahy was then be reacted with these aldehyde groups to form the hydrogel coating.

15 Antibodies could be coupled to this hydrogel in at least two ways. In one method, the carbohydrate groups in the Fc antibody region are oxidized to aldehydes by treatment with sodium metaperiodate. However, few antigen-binding fragments contain carbohydrate moieties useful for this purpose. Thus, a preferred method comprised modifying the pendant hydrazido groups of the hydrogel to a maleimido  
20 group by treatment with succinimidyl 4-(N-maleimido-methyl)cyclo-hexane-1-carboxylate (abbreviated SMCC; Pierce Chemicals). These maleimido groups can be reacted with the free thiol groups typically found in the C-terminal region of Fab' fragments, thereby coupling the Fab' fragments to the hydrogel.

Polymethacryloylchloride (abbreviated PMaCl) was prepared by radical  
25 polymerization of methacryloyl chloride (abbreviated MaCl) in dioxane under an inert atmosphere, as described in Jantas et al., J. Polym. Sci., Part A: Polym. Chem. 27:475-485 (1989).

A reaction mixture containing 21.1 mole % of MaCl, 78.1 mole % dioxane, and 0.8 mole % AIBN (azobisisobutyro-nitrile), was allowed to react for 24 hours at  
30 60°C with agitation. The PMaCl so produced remained in solution during the course of the reaction. The mixture was then diluted with twice the amount of dioxane used in the reaction and slowly added to an excess of hydrazine hydrate, to achieve a volumetric ratio of 2:5 for diluted PMaCl. The latter addition was carried out for about 30 minutes in an ice bath under a nitrogen atmosphere. The resulting mixture

was then stirred for about an hour at room temperature. The product PMahy was purified by evaporation of dioxane and the remaining unreacted hydrazine hydrate, followed by washing in distilled water. The washed product was then dialyzed in a SpectraPor dialysis membrane having a molecular weight cut-off of 3,500 daltons, to  
5 remove unreacted monomer.

The polymer so prepared was shown to have a molecular weight of about 26,000 as measured by gel permeation chromatography for the hydrochloride form. The concentration of polymer in solution in the hydrochloride form was estimated to vary between about 5% and 8% (w/v). It has been found that the polymer can be  
10 stored in aqueous solution at 4°C under a nitrogen atmosphere, for at least 5 months without undergoing a detrimental amount of spontaneous cross-linking.

Silica chips or glass or quartz microscope slides were cleaned with chromic acid, then treated with 5% APS/95% deionized water (v/v) for about fifteen minutes at room temperature. The APS-treated surfaces were rinsed with deionized water  
15 and absolute ethanol, and incubated in a vacuum oven which had been flushed at least three times with nitrogen, at 120°C for 1 hour. The resulting silanized surfaces were then soaked in 2.5% glutaraldehyde (E.M. grade from Polysciences) in 0.1M carbonate-bicarbonate buffer, pH 9.2, for two hours at room temperature.

Next, linear PMahy was reacted with the aldehyde groups on the treated chips  
20 to create a cross-linked polymer film with many unreacted hydrazido groups in the chains. This was done by dipping the treated chips in solutions of PMahy of between about 5% and 8% (w/v), pH 5.2, at a temperature between about room temperature and about 60°C, for a time sufficient to form a polymer film of thickness about 100 Å or less. The thickness of the hydrogel layer increases with time and temperature of  
25 incubation in the solution. It was found that optimal conditions for preparation of the film of 100 Å thickness or less, comprised incubating in 5% (w/v) PMahy for 2 hours at room temperature (about 25°C).

Next, the free hydrazido groups of the polymer film were modified by treatment with SMCC to provide reactive maleimido groups on the ends of the  
30 polymer side chains. This was done by immersing the PMahy-coated substrates in a solution of 0.19% (w/v) SMCC in dimethylformamide for about 1 hour at 25°C.

Following derivatization with SMCC, the hydrogel-coated surfaces were treated with a 1 mg/ml solution of Fab' fragments in phosphate buffer, pH 6.0, with 5 mM EDTA. The waveguide surface so prepared was shown to immobilize Fab'



molecules at a surface density of about  $1.4 \times 10^{-12}$  moles/cm<sup>2</sup>. Also the surface was able to immobilize Fab' fragments at their C-terminal thiol groups in a site-specific way. The thickness of the resulting polymer film was determined by ellipsometry to be about 100 Å, as was desired. This film thickness is much less than typical  
5 previous polymeric films, which have thicknesses of 0.35 to 25 µm (microns). The above-described method of preparing the PMahy polymers is superior to that described by von Kern et al. using polymethacryloylacid esters. Such esters suitable for reaction with hydrazine hydrate often have a molecular weight of 80,000 daltons or more, from which it is difficult to obtain a desirably thin film on the waveguide.

10 Finally, the Fab' fragments were coupled to the free maleimido groups pendant from the polymer-coated surface as follows. The prepared waveguide surface was incubated for 24 hours at 4°C in a solution containing the Fab' fragments at a concentration of  $1.5 \times 10^7$  molar, in a phosphate buffer with 5 mM EDTA (pH 6.0).

## 15 EXAMPLE II

### PREPARATION OF WAVEGUIDE SURFACE - AVIDIN-BIOTIN

This strategy was designed to exploit the very strong binding affinity of biotin for avidin (binding constant of around  $10^{15}$ ). An avidin coating was readily made by physical adsorption on a silica surface. The Fab' fragments were then conjugated  
20 with biotin to form biotin-Fab' conjugates, also referred to as biotinylated Fab' fragments or b-Fab' fragments. The biotin is coupled at specific location(s) on the Fab' fragments. The avidin coated surface is then treated with the b-Fab' fragments, so that the biotin binds to the avidin thereby immobilizing the Fab' fragment to the surface in a site-specific manner.

25 In actual experiments, the procedure was as follows. Chromic acid-cleaned silica surfaces were immersed in a solution of  $3 \times 10^{-6}$  M (molar) avidin for about 3 hours at room temperature. The surfaces were then washed several times in PBS to remove unadsorbed avidin.

Biotinylated Fab' conjugates were prepared from a solution of Fab' fragments  
30 in PBS (0.5-1 mg/ml), by addition of a sufficient amount of 4 mM biotin-HPDP in dimethyl-formamide to provide a 20-fold molar excess of biotin-HPDP. This mixture was incubated for 90 minutes at room temperature, and biotinylated Fab' fragments (abbreviated b-Fab') were purified by gel permeation chromatography with Sephadex G25 equilibrated in PBS.



An alternate method was used for biotinylating whole antibodies, in which biotin-LC-hydrazide was coupled to oxidized carbohydrate groups in the Fc region of the antibody. Mab designated 9-40 (a murine monoclonal IgG<sub>1</sub> antibody that binds fluorescein), was oxidized by incubation at a concentration of 1-2 mg/ml protein in 10 mM sodium periodate, 0.1 M sodium acetate pH 5.5 for 20 minutes at about 0°C. Glycerol was then added to a final concentration of 15 mM to quench the reaction, and the mixture incubated a further 5 minutes at 0°C. The oxidized Mab 9-40 was purified by gel filtration chromatography on Sephadex G25 equilibrated with 0.1M sodium acetate buffer pH 5.5, and then reacted with 5 mM biotin-LC-hydrazide for 2 hours at room temperature with agitation. Unreacted biotin-LC-hydrazide was removed using a Sephadex G25 column equilibrated in PBS.

Avidin-coated surfaces were immersed in a  $1.5 \times 10^{-7}$  M solution of b-Fab' fragments for about an hour at room temperature, followed by washing with PBS to remove unbound b-Fab' fragments. Optionally, polyethylene glycol (abbreviated PEG) was coupled to surfaces that were previously coated with the b-Fab' fragments, by immersion of the b-Fab'-coated surfaces in a solution of between about  $5 \times 10^{-8}$  and  $1 \times 10^{-7}$  M PEG. Unbound PEG was removed by washing in PBS.

The density of immobilized Fab' fragments obtained using the avidin-biotin coupling chemistry was about  $1.4 \times 10^{-12}$  moles per cm<sup>2</sup> (square centimeter).

### EXAMPLE III

#### PREPARATION OF WAVEGUIDE SURFACE - PEG-TYPE

In this method, the terminal hydroxyl groups of polyethylene glycol (abbreviated PEG) were converted to primary amine or hydrazide groups by reaction with ethylenediamine (abbreviated ED) or hydrazine (abbreviated HZ), respectively, to produce PEG-ED<sub>2</sub> or PEG-HZ<sub>2</sub>. The PEG molecules so modified were then coupled to APS-glutaraldehyde activated silica surfaces. One ED moiety on each PEG-ED<sub>2</sub> molecule couples to a free aldehyde group on the silanized-glutaraldehyde-treated waveguide surface. The other ED (or HZ, if PEG-HZ<sub>2</sub> is used) is then available to bind to an aldehyde moiety in a capture molecule (binding protein) such as an oxidized antibody or antibody fragment.

Monofunctional (PEG M2000, M5000) or difunctional (PEG 3400, PEG 8000, PEG 18,500) of the indicated molecular weights in daltons, were reacted with p-nitrophenyl chloroformate (abbreviated p-NPC; obtained from Aldrich Chemicals) in

solution in benzene. The mixture was agitated at room temperature for about 24 hours. Dry ethyl ether (less than 0.01 % water, purchased from J.T. Baker Chemicals) was used to precipitate PEG-(o-NP)<sub>2</sub> from solution. The precipitate was vacuum-dried overnight. Between about 50 % and about 100 % of PEG molecules  
5 were converted by this treatment to PEG-Onp, as determined by hydrolysis with 0.1N sodium hydroxide to release the p-nitrophenol groups. The absorbance at 402 nm was determined spectrophotometrically and a molar extinction coefficient of 18400 M<sup>-1</sup> cm<sup>-1</sup> used to determine the amount of conversion. The level of conversion depended somewhat on the molecular weight of the PEG of MPEG.

10 PEG-(o-NP)<sub>2</sub> was then dissolved in ethylenediamine and agitated gently for about 3 hours at room temperature. The PEG-(ED)<sub>2</sub> was then precipitated by addition of a sufficient amount of dry ethyl ether. The yellow PEG-(ED)<sub>2</sub> solution was decolorized by addition of 1 drop of 12N (normal) hydrochloric acid, and the precipitation with ethyl ether repeated twice more. The wet PEG-(ED)<sub>2</sub> was dried  
15 under vacuum overnight. Alternatively, in place of ethylenediamine, the PEG was derivatized with hydrazine to produce PEG-Hz<sub>2</sub>.

The modified PEG was coupled to silanized-glutaraldehyde-treated waveguide surfaces prepared as described in Example I. A solution of 24 milligrams (mg) of PEG-ED powder dissolved in 1.2 milliliters (ml) of 0.15M PBS pH 7.4 or in the same  
20 volume of 11 % potassium sulfate-sodium acetate buffer at pH 5.2. The prepared waveguide surfaces were immersed in the PEG-ED solution and incubated at 60°C for about 24 hours. The procedure using K<sub>2</sub>SO<sub>4</sub>-acetate buffer yielded a higher density of PEG molecules attached to the surface than that using PBS buffer. Antibodies or other binding proteins were immobilized to the PEG-coated waveguides as follows. A  
25 solution of about 3 mg/ml of antibody was dissolved in 0.15 M sodium acetate buffer, pH 5.2. A solution of equivalent weight of 50 mM sodium metaperiodate (NaIO<sub>4</sub>) was then added, and the reactants were agitated at room temperature for about an hour. Unreacted sodium metaperiodate was removed by passing the reaction mixture through a desalting column (type PD-10 from Pharmacia), which had been pre-  
30 equilibrated with the sodium acetate buffer.

The PEG-coated waveguides were then incubated with the oxidized antibody solution in the sodium acetate buffer, pH 5.2, for 3 days at 4°C, then rinsed to remove unbound antibody.

Waveguides prepared by each of the coating procedures of Examples I-III, as well as by prior art random-site coupling methods, were analyzed to determine levels of non-specific binding relative to total fluorescence and amounts of immobilized antibody and of available binding sites. Comparative results of these analyses are shown in Table I.

The data in Table I were obtained using fluorescein-BSA (BSA = bovine serum albumen) conjugates with an epitope density of nine (that is, approximately nine fluorescein molecules bound per BSA molecule), and anti-fluorescein antibodies (designated Mab 9-40, or Fab' 9-40 for fragments). A hybridoma cell line secreting this antibody was obtained from Professor E. W. Voss of the University of Illinois at Urbana-Champaign. In these experiments and those whose results are shown in FIGS. 7A, 7B, 8, 9A-9F, and 10A-10D, data acquisition and processing was accomplished using software supplied with the Photometrics Series 200.

Absolute antigen binding was determined by means of radiolabelled tracers or capture molecules. For example, radiolabelled BSA-FL<sub>9</sub> was allowed to be coupled with immobilized Fab' fragments for 5 or 60 minutes in phosphate buffer pH 7.3 at room temperature. The tracer concentration was  $1.5 \times 10^{-7}$  M. Three ml per sample of fluorescein-labelled BSA (BSA-FL<sub>9</sub>) at concentrations ranging from  $10^{-10}$  M to  $10^{-7}$  M was injected into the flow cell. The injection was performed over a five-minute interval. The spectrum at wavelength of 488 nm was taken and the bulk BSA-FL<sub>9</sub> was removed by flushing with PBS buffer. Three more spectra were taken, and the fluorescein peak from 513 to 517 nm was integrated. These values were set versus the log of BSA-FL<sub>9</sub> concentration in order to obtain the binding isotherm.

For measurements of radioactivity, the coated silica chips with either immobilized radiolabelled capture molecules or with labelled tracer molecules bound to unlabelled capture molecules, were washed thoroughly in a suitable buffer and counted on a gamma counter. <sup>125</sup>I-labelled antibodies or antigens were preferred for the radiolabelling, which was done using the Chloramine-T method (see Greenwood et al., Biochem. J. 89:114-123 (1963)).

The levels of non-specific absorption of antigen on waveguides prepared by site-specific coupling with avidin-biotin (Example II; Table I rows 7 and 8 from the top) or hydrogel (Example I; Table I bottom two rows) were considerably better than most of the prior art coupling methods, being typically 1-3% (Table I). The results

Table I  
Summary of Solid-Phase Immunoassays using Silica Substrates

Surface <sup>1</sup>	Antibody <sup>2</sup>	Coupling Chemistry	Total Binding <sup>3</sup> ( $\times 10^{-12}$ ) (moles/cm <sup>2</sup> )	Absolute Non-specific Binding <sup>4</sup> ( $\times 10^{-12}$ ) (moles/cm <sup>2</sup> )	Relative Non-specific Binding (%)	Immobilized Antibody <sup>5</sup> ( $\times 10^{-12}$ ) (moles/cm <sup>2</sup> )	Specific Activity <sup>6</sup> (%)
			( $\times 10^{-12}$ ) (moles/cm <sup>2</sup> )	( $\times 10^{-12}$ ) (moles/cm <sup>2</sup> )	(%)	( $\times 10^{-12}$ ) (moles/cm <sup>2</sup> )	(%)
Hydrophobic Silica (DDS)	Heat Treated IgG	Random	0.65	0.04	6.46	3.00	21.67
Hydrophobic Silica (DDS)	Acid Treated IgG	Random	0.67	0.07	9.70	2.20	30.45
Hydrophobic Silica (DDS)	Fab' Fragment	Random	0.37	0.25	69.00	1.30	28.46
Silica/APS/GLU	Acid Treated IgG	Random	0.55	0.21	38.18	3.75	14.67
Silica/APS/GLU/PEG	Oxidized IgG	Specific	0.56	0.11	19.64	2.06	27.18
Silica/APS/GLU/PEG	Oxidized IgG	Random	0.41	0.10	24.39	1.44	28.47
Silica/Avidin	Biotin-IgG	Specific	0.72	0.02	2.92	0.94	76.60
Silica/Avidin	Biotin-Fab	Specific	0.84	0.02	2.62	1.10	76.36
Silica/Avidin (with biotin-PEG)	Biotin-Fab	Specific	0.80	0.02	1.88	"	72.73
Silica/Hydrogel (preswollen)	Oxidized IgG	Specific	0.17	0.01	6.88	7.95	2.14
Silica/Hydrogel (preswollen)	Fab' Fragment	Specific	1.51	0.03	2.03	2.76	54.71

<sup>1</sup>Abbreviations: DDS - dichlorodimethylsilane; APS - aminopropylsilane; GLU - glutaraldehyde; PEG - polyethylene glycol (3400 MW); BSA - bovine serum albumin; IgG - intact immunoglobulin G; Fab' - antigen binding fragment with reactive thiol group; ND - not determined

<sup>2</sup>All immunoassays were performed with an IgG, monoclonal antibody (9-40) which binds fluorescein.

<sup>3</sup>Amount of <sup>125</sup>I-Fluorescein-BSA which bound to silica substrate.

<sup>4</sup>Amount of <sup>125</sup>I-BSA which bound to silica substrate.

<sup>5</sup>Amount of <sup>125</sup>I-9-40 immobilized on silica substrate.

<sup>6</sup>Percent of immobilized active sites which bound antigen molecules.



Table II  
Summary of Solid Phase Immunoassays Using Silica Substrates Covered with  
Hydrogel with Maleimido Reactive Groups

Antibody	Binding Constant $pK_o$	Immobilized Antibody	Total hCG Binding in 5 min	Total hCG Binding in 60 min	Antibody Activity	Absolute Non-specific Binding in 5 min (BSA)	Relative Non-specific Binding in 5 min (BSA)	Absolute Non-specific Binding in 60 min (BSA)	Relative Non-specific Binding in 60 min (BSA)
		( $\times 10^{-12}$ mol/cm <sup>2</sup> )	( $\times 10^{-12}$ mol/cm <sup>2</sup> )	( $\times 10^{-12}$ mol/cm <sup>2</sup> )	(%)	( $\times 10^{-12}$ mol/cm <sup>2</sup> )	(%)	( $\times 10^{-12}$ mol/cm <sup>2</sup> )	(%)
Fab' from Anti-hCG-A	8.85	$1.39 \pm 0.07$	$0.62 \pm 0.03$	$0.81 \pm 0.03$	$58.3 \pm 0.8$	$< 0.01$	$< 0.97$	$< 0.02$	$< 2.47$
Fab' from Anti-hCG-B	7.89	$1.29 \pm 0.06$	$0.51 \pm 0.02$	$0.67 \pm 0.03$	$51.9 \pm 0.1$	$0.02 \pm 0.01$	$3.85 \pm 1.81$	$0.04 \pm 0.01$	$5.91 \pm 1.22$
Fab' from Anti-hCG-C	8.70	$0.74 \pm 0.04$	$0.18 \pm 0.01$	$0.41 \pm 0.02$	$55.4 \pm 0.3$	$< 0.01$	$< 6.22$	$0.03 \pm 0.01$	$7.21 \pm 2.09$
Fab' from Anti-hCG-D	8.00	$1.45 \pm 0.06$	$0.58 \pm 0.02$	$0.75 \pm 0.03$	$51.4 \pm 0.3$	$< 0.01$	$< 1.20$	$< 0.02$	$< 3.21$
Fab' from Mouse IgG	-	$2.50 \pm 0.10$	$0.04 \pm 0.01$	$0.06 \pm 0.02$	$2.4 \pm 0.7$	-	-	-	-



also indicated that non-specific binding to the avidin-coated waveguide was acceptably low for analyte molecule concentrations of less than about  $10^{-5}$  M, without a wash step.

5 The percentage of immobilized molecules that were active (able to bind analyte) was also considerably higher for avidin-biotin and for hydrogel coupling chemistries, being in the range of 50% to 75% for Fabs. The results for IgG capture molecules coupled by heat treatment, acid treatment or by oxidation, indicated that only a low percentage of the IgG binding sites were active (Table I rows 1,2,4, 5,6,10 from the top).

10 The row labelled silica-avidin with biotin-PEG, represents data obtained with the further refinement of preloading the surface (after attachment of the capture molecules) with biotin-PEG conjugates. This was done to passivate potential non-specific binding regions. However, the improvement obtained with biotin-PEG preloading was not large.

15 For the experiments whose results are shown in Tables II and III, Fab' fragments derived from a murine anti-human chorionic gonadotrophin (anti-hCG) monoclonal IgG antibody were used. The parent monoclonal antibody was purified as described by van Erp et al. *J. Immunol. Methods*, 140:235-241 (1991). This mouse antibody, termed anti-hCG-A, is directed against a portion of the  $\beta$ -subunit of hCG  
20 (provided by Organon-Teknika of Boxtel, Netherlands). The whole monoclonal antibody anti-hCG-A was used in the experiments whose results are depicted in FIGS. 7A, 7B, 8, 9A-9F, and 10A-10D.

F(ab')<sub>2</sub> fragments were produced by digestion with pepsin using the procedure described by Grey and Kunkel, "H Chain subgroups of myeloma proteins and normal  
25 7S globulin," *J. Exp. Med.* 120:253-266, 1964. Following digestion, F(ab')<sub>2</sub> fragments were reduced to Fab' fragments using dithiothreitol (DTT). Specifically, 33 mg of purified antibody and 1 mg pepsin (Sigma) were dissolved in 0.1 M sodium acetate buffer (pH 4.2) and the digestion was carried out at 37°C for 16 hours. The digestion was terminated by adjusting the pH of the reaction mixture to 8.0 with 2 M  
30 tris base. The F(ab')<sub>2</sub> fraction was separated by gel permeation chromatography (Superdex Hiload, Pharmacia) using phosphate-buffered saline (PBS), pH 7.7, as eluent. Fab' fragments were prepared by reducing the F(ab')<sub>2</sub> fragments (1 mg/ml) with 1.75 mM DTT and 3.5 mM ethylenediamine tetraacetate (EDTA) in 0.17 M tris buffer (pH 7.4) for 45 minutes at room temperature. After reduction, excess DTT

was removed by gel permeation chromatography using a Sephadex G-25 column (Pharmacia) equilibrated in 0.1 M sodium phosphate buffer (pH 6.0) containing 5 mM EDTA.

Table III

Summary of Solid Phase Immunoassay Using Silica Substrates with Adsorbed Avidin and Biotinylated Fab' Fragments

Antibody	Immobilized Antibody	Total hCG Binding	Specific Activity	Absolute Non-specific Binding (BSA)	Relative Non-specific Binding (BSA)
	( $\times 10^{-12}$ mol/cm <sup>2</sup> )	( $\times 10^{-12}$ mol/cm <sup>2</sup> )	(%)	( $\times 10^{-12}$ mol/cm <sup>2</sup> )	(%)
Fab' from Anti-hCG-A	$1.19 \pm 0.02$	$1.22 \pm 0.01$	$100 \pm 5$	$0.05 \pm 0.02$	$4.20 \pm 0.02$
Fab' from Anti-hCG-B	$1.40 \pm 0.05$	$1.38 \pm 0.07$	$98 \pm 9$	$0.05 \pm 0.01$	$3.57 \pm 0.02$
Fab' from Anti-hCG-C	$2.24 \pm 0.02$	$1.10 \pm 0.03$	$49 \pm 3$	$0.05 \pm 0.02$	$2.32 \pm 0.01$
Fab' from Anti-hCG-D	$1.59 \pm 0.02$	$1.24 \pm 0.01$	$78 \pm 2$	$0.05 \pm 0.005$	$3.14 \pm 0.01$
Fab' from Mouse IgG	$1.25 \pm 0.02$	$0.03 \pm 0.003$	$2.4 \pm 0.05$	$0.09 \pm 0.03$	$7.20 \pm 0.03$

In the experiments whose results are presented in Tables II and III, the specific binding values were determined using hCG labelled with <sup>125</sup>I as described for Table I, while <sup>125</sup>I-labelled BSA was used to measure the non-specific binding. In both Tables II and III, the immobilized antibody was anti-hCG A.

The fluoro-immunoassays of FIGS. 7A, 7B, 8, 9A-9F, and 10A-10D and Tables I-III were performed using an interfacial fluorometer constructed at the University of Utah. Silica waveguides with the appropriate respective immobilized antigens were placed in the dual-channel flow-cell of FIGS. 3A and 3B. The two channels were used for sample and reference measurements, as described with respect to FIGS. 4A-C. The light source was the 514.5 nm emission of an air-cooled argon-ion laser. The laser beam was split into two parallel beams, which were focused with lenses into the two channels of the waveguide. Fluorescence emission was recorded

from 520 to 620 nm using a monochromator connected to a computer-controlled CCD camera. The fluorescence spectrum was integrated from 560 nm to 600 nm to improve the signal-to-noise ratio.

FIGS. 7A, 7B and 8 are charts depicting fluorescence intensity data obtained using two alternate formats for performing a fluorescence immunoassay to detect an antibody. In these experiments, the detection of antibodies to human chorionic gonadotrophin (abbreviated hCG) was used as a model to determine which format provided the greatest sensitivity. It will be evident that the methods described could be adapted to the detection of any desired antibody in biological fluids such as plasma or serum, for example the detection of antibodies to proteins of viral and bacterial pathogens, depending only on obtaining the necessary antigen for use as the capture molecule.

For purposes of the tests shown in FIGS. 7A, 7B and 8, the antibody to be detected (the analyte) was chosen to be a monoclonal antibody (designated anti-hCG-A) to an hCG antigen (the latter designated hCG-A). The data of FIG. 7A were obtained with whole hCG molecules serving as the capture molecules (the antigen or analyte binding molecule) in the assay. The data of FIG. 7B were obtained using an oligopeptide constructed to selectively bind the anti-hCG-A antibody, as the capture molecules. Oligopeptides suitable for this purpose for any known antigenic analyte molecule analyte can be obtained using the methods of Geysen et al. as disclosed in Patent Publication Nos. WO 86/86487 and U.S. Patent No. 4,708,871, as well as in the scientific literature. To attach the necessary fluorescent dye, either the N-terminus of the oligopeptide was modified to provide an amino group for amino-reactive dyes, or the C-terminus was modified to provide a cysteine thiol group for thiol-reactive dyes). Preferably also, the complete oligopeptide sequence is of length sufficient that the attached dye is spaced from the binding site by at least two or three residues.

In the experiments of both FIGS. 7A and 7B, the tracer was a goat anti-mouse IgG labelled with tetramethylrhodamine (abbreviated TMR). For both assay formats, the capture molecule was biotinylated as described in Example II and immobilized on an avidin-coated silica substrate. The test antibody, anti-hCG A, was premixed with the tracer (goat anti-mouse IgG-TMR) in the test solution.

As will be understood by those in the art for a sandwich fluoroimmunoassay, the anti-hCG A antibody bound to the immobilized capture molecule, and the goat-anti-mouse IgG-TMR tracer in turn bound to the mouse anti-hCG A antibody. In this

way a fluorescent sandwich formed on the substrate surface with the TMR-portion of the tracer molecule being held within the region of evanescent excitation.

The data of FIGS. 7A and 7B were obtained with the following protocol. Different concentrations of anti-hCG A were premixed with the tracer antibody (concentration fixed at  $10^{-8}$  M) and injected into the sample channel. A  $10^{-8}$  M concentration of tracer antibody was also injected into the reference channel as a control. The fluorescence intensity of the sample channel was plotted vs. anti-hCG A concentration and the fluorescence intensity of the reference channel was also plotted on the same set of axes (this is really a plot of the non-specific binding of the tracer antibody vs. time, since no anti-hCG A was injected into the reference channel).

FIGS. 7A and 7B show the results for a sandwich assay format following the binding of anti-hCG A to immobilized hCG and to the oligopeptide, respectively. Figure 8 shows the corresponding fluorescence enhancements for both cases. The data from FIGS. 7A and 7B were normalized for background fluorescence and replotted as fluorescence enhancement ( $F_{\text{sample}}/F_{\text{reference}}$ ) versus log analyte concentration. The response curve was similar for both of the immobilized antigens (whole hCG and oligopeptide antigen) over a range of antibody concentrations from  $10^{-13}$  M to  $10^{-10}$  M. However, whole hCG gave better precision.

It is also evident from FIGS. 7A, 7B and 8 that analyte levels (anti-hCG A) as low as  $10^{-13}$  molar were detectable with the assay. In a further embodiment, the tracer antibody concentration is reduced to  $10^{-10}$  M or less. This is expected to reduce background fluorescence due to non-specific adsorption of the tracer antibody and thereby further improve the sensitivity to  $10^{-14}$  M or better.

FIGS. 9A-9C depicts data obtained using an antibody as the capture molecule to detect an antigen, in a sandwich-type assay. As mentioned previously, two different antibodies are employed in a sandwich immunoassay — an immobilized capture antibody and a labelled tracer antibody in solution. Since the capture antibody and the tracer antibody must bind to distinct regions of the antigen, two different monoclonal antibodies which bind to different epitopes on the antigen are typically used in such assays. In addition to the anti-hCG-A, three other monoclonal anti-hCG antibodies (anti-hCG-B, anti-hCG-C and anti-hCG-D, respectively) were obtained from Organon Teknika which bound to different epitopes than did anti-hCG-A. Since only anti-hCG A is specific to hCG (the others also bind to certain hormones related



to hCG), only six of the twelve possible pairwise combinations of antibodies provide strict selectivity for hCG.

FIGS. 9A-F depict results obtained with different pairwise combinations, with Fab' fragments prepared from anti-hCG A (Fab'-A) and immobilized to waveguides using the avidin-biotin coupling chemistry. Fab' fragments prepared from anti-hCG B, anti-hCG C and anti-hCG D were labeled with tetramethylrhodamine for use as tracer antibodies (designated Fab'-B, Fab'-C and Fab'-D, respectively). FIGS. 9A and 9B show results with Fab'-B as the tracer molecule. FIGS. 9C, 9D show results obtained using Fab'-C as the tracer molecule. FIGS. 9E, 9F show results obtained using Fab'-D as the tracer molecule. Presently, Fab'-B and Fab'-C are preferred for use as tracers in an hCG assay.

An alternate format used a converse protocol, that is, Fab'-A as the tracer molecule and Fab'-B, -C or -D as the capture molecule. However, the format using Fab'-A as the capture antibody was generally superior in sensitivity. It can be seen from FIG. 9B that hCG concentrations as low as  $10^{-12}$  M could be detected by the assay with Fab'-A as capture molecule.

FIGS. 10A-D show data obtained from a competition or displacement assay. Fab'-A fragments were immobilized to waveguides using either the avidin-biotin chemistry (FIGS. 10A, 10B) or the hydrogel coupling chemistry (FIGS. 10C, 10D). The immobilized Fab'-A fragments were preloaded with the tracer oligopeptide at a concentration of  $10^{-8}$  M. Increasing concentrations of hCG were added to one channel of the flow cell (sample) and PBS buffer was added to the other (reference). For each coupling chemistry, the raw fluorescence intensities of the sample and reference channels are shown in the panels on the left (10A & 10C) and the percent of full-scale fluorescence (in the absence of hCG) are shown in the panels on the right (10B & 10D). The latter values were normalized for the change in reference fluorescence. Standard errors were plotted for all data points, but in some cases were smaller than the plot marks.

At present the sandwich immunoassay is preferred for several reasons. First, detection of concentrations down to at least 0.1 picomolar can be demonstrated, as compared to picomolar concentrations for the competitive assay. Also, the instant sandwich immunoassay was capable of detecting concentrations ranging over five logs - from  $10^{-8}$  M to  $10^{-13}$  M. Thus, a single assay formulation using the sandwich



procedure could serve for a variety of applications where different detection limits are required.

A further embodiment of coating chemistry, and one which at present is highly preferred, provides for photo-activated coupling of the binding moiety (Fab fragment, antibody or whatever) to the waveguide surface. By combining the photoactivation process with localized irradiation (for example, by masking), it is possible to sequentially couple different binding species to different regions of the waveguide surface. In this way, a waveguide surface patterned with patches each of a different capture molecule species (Fabs or Fab fragments) can be conveniently produced, without need for walls between the different species. For the present type of evanescent sensor, the elimination of unnecessary walls can significantly improve the sensitivity of the device by reducing background and enhancing evanescent field strength. In a highly preferred embodiment, the coating chemistry also "passivates" the surface, that is, inhibits nonspecific binding of the fluorescent tracer, and thus reduces the background signal.

A presently preferred coating is a type of compound referred to herein as a "block copolymer", comprising at least one hydrophilic block containing polymerized hydrophilic residues (polyethylene oxide, "PEO"), adjacent at least one hydrophobic block containing polymerized hydrophobic residues (polypropylene oxide, "PPO"). A subclass of such compounds referred to herein as "triblock copolymers" or "TBCPs", comprise a hydrophobic block flanked by hydrophilic blocks. A series of TBCPs are commercially available from BASF Corporation under the tradename PLURONICS. An example of a presently preferred compound is known generally in the literature as PLURONICS F108 or "PF108"; it has a molecular weight (MW) about 14,600 and the general formula  $(\text{PEO})_x(\text{PPO})_y(\text{PEO})_x$ , where  $x = 129$  and  $y = 56$ . In block copolymers, the hydrophobic PPO segment tends to adsorb strongly to plastics including polystyrene, leaving the PEO side-arms in a relatively mobile state. Block copolymers have the general property of inhibiting non-specific protein adsorption, while providing hydrophilic side chains useful to attach proteins, including Fabs or Fab fragments.

Also, while the present description is primarily with reference to PLURONICS- type compounds, it is within contemplation that other polymeric compounds having hydrophilic segments and hydrophobic segments and offering pendant OH groups for attachment of proteins or photo-activated linkers will be

useful. As known in the art, these include SEPHAROSE-type materials and other polysaccharides. Also, block copolymers having polyurethane segments as the hydrophilic block may be useful.

Referring to FIG. 16, a general procedure for preparing a patterned polystyrene waveguide is as follows. First, a waveguide surface 700 coated with PF108 molecules 702 is prepared. Next, the free PEO chain ends 704 of the PF108 molecules 702 in a selected region of the waveguide are derivatized in a photo-activated coupling reaction with a photoaffinity crosslinker 706. Suitable crosslinkers are heterobifunctional reagents which have a photo-activatable group conjugated to a reactive functional group such as isothiocyanate, succinimide or maleimide. Upon irradiation with light beam 701 of the appropriate wavelength (generally in the ultraviolet region), the photo-activatable groups of the crosslinker 706 react to covalently bind to the free PEO chain ends 704. A mask 712 confines the irradiation to a first region 714 of the waveguide. The result is waveguide surface having reactive functional groups useful to bind Fab' fragments only in the first region 714. Next, the waveguide surface is incubated with a solution of Fab' fragments of a first species (FAB 1 720 in FIG. 16) for a time sufficient to allow the binding of Fab' fragments to the derivatized region to go to completion. The unreacted Fab' fragments are then washed off, and the process of photo-activated derivatization is repeated for a second region of the waveguide, followed by incubation with a second species of Fab' fragment.

For coupling of Fab' fragments to a waveguide region derivatized with free maleimido groups (procedure of FIG. 16), incubation with a solution of Fab' fragments can be performed substantially as described for the PMahy coating.

In an alternate embodiment, the Fab' fragments 720 are coupled to the crosslinker 706 before the crosslinker is photo-reacted with the PEO chain ends 704 (FIG. 17). This embodiment is presently preferred because the surfaces thus prepared are capable of binding larger levels of analyte plus tracer per unit area than those prepared according to the protocol of FIG. 16.

Suitable photoaffinity crosslinkers include aryl azides (amine-to-amine linkage), fluorinated aryl azides (C-H bond-to-amine linkage), and benzophenones (C-H bond-to-amine linkage or C-H bond-to-thiol-linkage, depending on the specific compound). Examples of each type are shown in FIGS. 18A-18D, along with the corresponding photo-activated coupling reaction. Presently, benzophenones providing a C-H bond-

to-thiol linkage are preferred, as these can be used to achieve site-specific coupling to Fab' fragments. Either the iodoacetamide or the maleimide derivatives of benzophenone ("BPIA" and "BPM", respectively) can achieve this purpose. At present BPM is preferred, as it exhibits a higher degree of specific binding and a lower degree of non-specific adsorption. This is because the coupling occurs via the C-terminal thiol groups of the Fab' fragments, as described previously herein for the PMahy coating. Other photo-affinity crosslinkers providing free maleimido groups may be equally suitable.

In the photocoupling process, the amount of crosslinker coupled to the PF108 depends on the duration and intensity of the irradiation, the concentration of crosslinker molecules, etc. These variables can easily be tested and optimized to find parameters which will achieve a desired level of crosslinker and/or Fab' protein coupled to the waveguide surface. Generally, a Fab' concentration of about 0.5 mg/ml to about 1 mg/ml, and a 20-fold molar excess of crosslinker, are useful in the processes of FIGS. 16 and 17.

Table IV shows comparative data on the levels of specific binding and non-specific binding obtained for the crosslinkers BPM vs. BPIA, for surfaces which are uncoated or coated with one of four different TBCPs, and for the procedure of FIG. 16 vs. that of FIG. 17. The TBCPs are PF108, and three others designated by the tradenames PP105, PF68, and PF88, also available from BASF. The respective PEO/PPO/PEO ratios and molecular weights of these compounds are 37/56/37 (PP105, MW=6500), 76/30/76 (PF68, MW=8400), and 104/39/104 (PF88, MW=11,400).

As a model system, Fab' fragments derived from the 9-40 anti-fluorescein antibody were used as the capture molecules, with fluorescein-conjugated BSA representing the analyte. The BSA was radioactively labelled. Specific binding was determined as the binding of the fluorescein-BSA-conjugate, while nonspecific binding was determined from binding of native (unconjugated) BSA.

From the data in Table IV, it is evident that the degree of non-specific binding was significantly lower for the PF108 and PP105 coatings than for PF68 and PF88. For this reason, PF108 and PP105 are presently preferred BCPs. In general, among TBCP compounds, those exhibiting better resistance to non-specific binding (and thus presently preferred) are those having PPO segments of length about 45-50 residues or

Table IV  
Specific and Non-Specific Binding of Antigen to  
Antibodies Immobilized to Polystyrene Using Photo-  
Affinity Cross-Linking Reagents

Experiment No.	Pluronics Coating Time	Cross- linker	Fab' Concen- tration	Irradi- ation Time	Specific Binding (SB)	Non- Specific Binding (NSB)	Relative NSB (NSB/SB)
			(Molar)	(min.)	(mol. cm <sup>-2</sup> )	(mol. cm <sup>-2</sup> )	(Percent)
1. Fab	None	BPIA-Fab	5.00e-6	30	5.83e-13	6.74e-14	11.6
2. Fab	None	PS-BPM	5.00e-6	30	9.20e-13	3.71e-13	40.0
3. Fab	None	BPM-Fab	5.00e-6	30	1.00e-12	7.04e-14	7.0
4. PF108-Fab	24 hours	BPIA-Fab	5.00e-6	30	1.01e-12	1.95e-13	19.0
5. PF108-Fab	24 hours	PL-BPM	5.00e-6	30	1.74e-13	2.02e-14	11.6
6. PF108-Fab	24 hours	BPM-Fab	5.00e-6	30	1.35e-12	1.48e-13	11.0
7. PF108-Fab	24 hours	BPM-Fab	1.00e-6	30	3.79e-13	2.08e-14	5.5
8. PF108-Fab	24 hours	BPM-Fab	5.00e-7	30	3.40e-13	1.86e-14	5.5
9. PF108-Fab	24 hours	BPM-Fab	1.50e-6	20	2.92e-13	3.43e-15	1.2
10. PF108-Fab	24 hours	BPM-Fab	1.50e-6	10	3.86e-13	5.66e-15	1.5
11. PP105-Fab	24 hours	BMP-Fab	1.50e-6	10	3.66e-13	<1e-15	<0.3
12. PP105-Fab	24 hours	BPIA-Fab	1.50e-6	10	1.07e-12	3.61e-14	3.4
13. PP105-Fab	24 hours	BPIA-Fab	5.00e-6	30	6.78e-13	3.87e-15	0.6
14. Silica- MSil5000-Fab		BPIA Fab	5.00e-6	30	1.17e-13	6.66e-15	5.7
15. Silica- MSil5000-Fab		BPIA Fab	1.50e-6	30	1.66e-13	Not Determined	-

In all experiments except Nos. 14 & 15, the substrate was a polystyrene surface. In expts. 1-3, there was no PLURONICS coating. In experiments 4-13, the surface was coated for 24 hours with a 4% w/v aqueous solution of the indicated PLURONICS compound. In experiments 1, 3, 4, and 6-15, the Fab' was coupled first to the crosslinker and the complex then photo-crosslinked to the substrate. In experiments 2 and 5, the crosslinker was first photo-coupled to the surface, then incubated with Fab. The concentration of crosslinker used was a 20-fold molar excess of the Fab' concentration.

more. Desirably, the level of non-specific binding should be no more than about 10%, and preferably below about 1%-2%, of the level of specific binding. Alternately, or in

addition, it is desirable that the absolute amount of non-specific binding be in the range below about  $5 \times 10^{-14}$  and preferably below about  $5 \times 10^{-15}$ . Neither the hydrophilic/lipophilic balance of the TBCP, the total MW, or the molecular weight ratio of PEO to PPO in the compound, appear to be as important as the length of the PPO segment in selecting TBCPs with good efficiency in inhibiting non-specific binding.

Table V contains comparative data concerning the effect of different TBCP coating times on non-specific binding to PF108-coated polystyrene waveguides. As can be seen, the levels of non-specific binding achieved were indistinguishable for coating times at least as short as 10 minutes up to at least as long as 24 hours.

From the data in Tables IV and V, it appears that the best results are obtained PF108 and PP105 and with Fab' concentrations of  $1.5 \times 10^{-6}$ M.

TABLE V

Effects of Pluronics Coating on the Non-Specific Binding of BSA and Fluorescein-BSA to Polystyrene

Experi- ment No.	Pluronics	Pluronics Coating Time	Fluorescein- BSA	BSA
			(mol. cm <sup>-2</sup> )	(mol. cm <sup>-2</sup> )
1.	None	--	6.05e-12	8.37e-13
2.	F108	24 hours	5.46e-14	8.13e-15
3.	None	--	1.96±0.04e-12	1.53±0.06e-12
4.	P105	24 hours	<1e-15	<1e-15
5.	F68	24 hours	1.68±0.36e-13	0.91±0.32e-13
6.	F88	24 hours	1.03±0.30e-13	0.32±0.17e-13
7.	F108	10 min	<1e-15	<1e-15
8.	F108	30 min	<1e-15	<1e-15
9.	F108	60 min	<1e-15	<1e-15
10.	F108	180 min	<1e-15	<1e-15
11.	F108	24 hours	<1e-15	<1e-15

In addition to the TBCPs discussed above, diblock copolymers of PEO/PPO (DBCPs) will also be effective as waveguide coatings to inhibit non-specific binding.



Here as with the TBCPs, those compounds having PPO segments of sufficient length, generally greater than about 40-45 residues, will be more effective. At present, TBCPs are preferred over DBCPs, because the PEO blocks are largely responsible for the non-specific-binding-inhibition properties of these compounds.

5           The above-described coupling scheme is very effective with a polystyrene waveguide, but less useful with silica-based substrates such as quartz, glass, and silicon oxynitride. The latter are of particular interest from the standpoint of the development of "integrated waveguide chip" biosensors. Therefore, in an alternate embodiment of the photocoupling method for a silicon oxynitride waveguide, a silica-affinic agent having a  
10   silyl group free to react with silica in the surface is substituted for PF108. Examples of useful silanizing (silica-affinic) agents are methoxypoly(ethyleneglycol) trimethoxysilane ("PEG-silane") of molecular weight around 3500-5000, aminopropyl-triethoxysilane (APS), and dichlorodimethylsilane (DDS). In this embodiment, PEG-silane and APS are presently preferred for their better non-specific-binding-inhibition properties.

15           In an alternate embodiment for silica-based substrates, the surface is coated with the silanizing agent. The silanized surface is then modified in a selected region with a photo-affinity crosslinking agent, in a manner similar to that described for the polystyrene/PF108 procedure. With APS as the base coating, a suitable crosslinker would be an aryl azide (FIGS. 18A-18D) which would crosslink the amino group in APS  
20   to amine groups in the Fab. However, this protocol would not provide site-specific attachment of the Fab to the waveguide surface, since Fabs have plural amine groups. Alternately, to achieve site-specific Fab attachment, PEG could be coupled to the APS as described previously herein, and a benzophenone crosslinker used to attach the Fab fragment to the PEG. The chemistry for the latter process would be similar to that for  
25   site-specific coupling of Fab fragments to PF108 with benzophenone crosslinkers.

Next, a selected Fab' fragment is coupled to the free functional group on the crosslinker. The remainder of the process (repetition of the above steps for different species of Fab') is similar to that for polystyrene waveguides.

30           Still another embodiment useful for silica-type waveguides involves undercoating the waveguide with a silica-affinic agent that produces a hydrophobic surface, such as DDS (dichlorodimethyl silane), followed by coating with a block copolymer such as PF108. The PF108 will adhere to the hydrophobic first coating, and the remainder of the coupling process is substantially as described for the polystyrene waveguide. Similar

considerations of the relative and/or absolute levels of non-specific binding apply in selecting preferred TBCP compounds and silica-affinic coating agents.

The patterning processes described above require a suitable light source for localized crosslinker photo-activation. As described above, localized photo-coupling may  
5 be performed with an incoherent light source such as a xenon lamp with lens and a mask (FIG. 15). A mercury vapor lamp with a 300 nm bandpass filter and a mask are another example of a useful incoherent light source. Alternatively, a coherent UV light source such as an argon ion laser may be used in combination with a translation stage. In the latter embodiment, a mask may or may not be needed to achieve the desired localized  
10 irradiation.

While many of the preceding experimental examples and results were obtained using hCG antigen/anti-hCG antibody and fluorescein/anti-fluorescein antibody systems, it will be understood by those skilled that the apparatus and the biosensor, as well as the site-specific waveguide-coupling methods and assay formats, all are applicable to assays  
15 for any antigen or antibody for which the requisite reagents such as appropriate capture molecules can be obtained, without undue experimentation. It will further be understood that while tetramethyl-rhodamine, fluorescein, and cyanine dyes are specifically mentioned as useful for labelling of tracer molecules, the apparatus and methods can also be useful with other fluorescent dyes capable of being conjugated to the desired tracer  
20 molecule.

Also, while the novel subject matter of this application is described herein primarily with respect to the apparatus in which excitation is by an evanescent field, the evanescent field is produced by directing a light beam into the edge or end of a waveguide, and the resulting fluorescence is directly collected from the evanescent zone  
25 (e.g., not via evanescent coupling back into the waveguide), the usefulness of many elements of both the optical and chemical portions of the subject matter is not so limited. Many elements in the instant subject matter will also be useful in alternate configurations of evanescent-light biosensors. One such alternate configuration is that in which the tracer molecules are excited by a non-evanescent light source, and the fluorescence is  
30 collected as evanescent light that propagates through the waveguide and is collected at the edge or end. Another such alternate configuration provides evanescent field excitation via a waveguide illuminated from the edge or end, with collection of fluorescent light by evanescent penetration back into the waveguide.

It will further be recognized that various modifications and substitutions may be made to the apparatus and the biosensor as described herein, without departing from the concept and scope of the invention.

The Claims

1. Apparatus for homogeneous solid-state fluorescence immunoassays, comprising a biosensor having  
a planar waveguide having first and second parallel plane surfaces with an edge extending  
5 between said first and second surfaces, said edge having a receiving region for receiving light, and at least one of said surfaces having a plurality of capture molecules immobilized thereon, said capture molecules each having a binding site which selectively binds an analyte, and  
a semi-cylindrical lens integrally adapted to said waveguide adjacent said  
10 receiving edge.
2. The apparatus of Claim 1, further including:  
a light source providing a light beam in a desired wavelength range and positioned to aim  
said beam into said lens;  
15 one or more reservoirs formed on at least one of said waveguide surfaces, each said reservoir configured to contain a sample solution comprising a buffer, a plurality of molecules of at least one said analyte, and a plurality of tracer molecules capable of emitting fluorescent light upon stimulation with evanescent light resulting from said beam; and  
20 detection means disposed for directly detecting a fluorescence signal consisting essentially of said fluorescent light, and including lens means positioned to collect said fluorescent light as it impinges on a plane parallel to and displaced from said surfaces.
- 25 3. The apparatus of Claim 2, having a plurality of spaced patches of said immobilized capture molecules, and wherein said detection means is configured to simultaneously separately collect a plurality of fluorescence signals each corresponding to an individual one of said patches.
- 30 4. The apparatus of Claim 3, wherein said detection means comprises an imaging detector having a plurality of photodetection elements spaced from each other in said displaced parallel plane, and lens means positioned to focus each of said fluorescence signals onto a respective one of said photodetection elements.

5. The apparatus of Claims 1 or 2, wherein said waveguide and said lens are formed of an optical plastic and molded as a single piece.

6. The apparatus of Claims 1, 2 or 5, wherein said semicylindrical lens has a focal region oriented to aim a sheet beam at a non-zero angle into said receiving edge, said angle being below a critical angle providing total internal reflection of light at a boundary between said waveguide and an aqueous medium.

7. The apparatus of Claims 1 or 6, wherein said waveguide has a serrated portion forming a terminal edge, said terminal edge being located at an end opposite said receiving edge and said semicylindrical lens.

8. The apparatus of Claims 1, 2, 3, or 4, wherein said waveguide is an optical substrate according to Claim 9.

15

9. The apparatus of Claims 2, 3, or 4, wherein said waveguide is an optical substrate according to Claim 10 and having a waveguide thickness, and further including beam shaping means located between said light source and said semicylindrical lens for shaping the light beam into a sheet beam having a thickness generally less than about twice said waveguide thickness.

20

10. An optical substrate useful for a solid state assay in which a capture molecule useful to specifically capture a corresponding analyte for detection of said analyte by means of a light signal is coupled to the optical substrate, comprising an optical surface having a region coated with a coating providing an acceptably low amount of nonspecific binding to the substrate, the coating being selected from the group consisting of: hydrogel formed of polymethacryloyl polymers, a silyl-derivatized polyethyleneglycol, avidin, and block copolymers, wherein said block copolymers comprise at least one hydrophobic polymer block consisting essentially of hydrophobic residues adjacent at least one hydrophilic polymer block consisting essentially of hydrophilic residues.

25

30



11. The optical surface of Claim 10, further including one or more species of capture molecules covalently coupled to said coated region, each said one or more species being constructed to specifically bind a corresponding analyte.

5           12. The optical surface of Claim 10, further including a crosslinking agent having a photo-responsive coupling moiety and a protein coupling moiety operable to covalently bind a capture molecule, said crosslinking agent selected from the group consisting of: aryl azides, fluorinated aryl azides, and benzophenones; wherein said crosslinking agent is covalently coupled to said coating via said photo-responsive coupling moiety.

10           13. The optical surface of Claim 12, further including one or more species of capture molecules covalently coupled to said coated region via said protein coupling moiety of said crosslinking agent, each said one or more species being constructed to specifically bind a corresponding analyte.

15           14. The optical substrate of Claims 10, 12 or 13, wherein said coating is a block copolymer having the general formula  $(\text{PEO})_x(\text{PPO})_y(\text{PEO})_z$ , where PEO = polyethylene oxide and PPO = polypropylene oxide, and wherein each of x, y and z may range between about 30 and about 150.

20           15. The optical substrate of Claims 12 or 14, wherein said capture molecules each have a thiol group reactively disposed therein, and wherein said capture molecules are coupled to said coating via said thiol group.

25           16. The optical substrate of Claims 12 or 14, wherein said crosslinking agent is benzophenone maleimide.

30           17. The optical substrate of Claims 10, 12 or 16, wherein said optical substrate is a silica-based material, and further including an undercoating applied to said region between said optical surface and said coating, said undercoating comprising a silica-affinic reagent having at least one reactive silyl group.

          18. A method of making an optical substrate according to Claim 10, including the steps of:

coating a region of an optical substrate with a coating selected from the group consisting of: hydrogel formed of polymethacryloyl polymers, avidin, methoxypoly(ethyleneglycol)trimethoxysilane, and block copolymers, wherein said block copolymers comprise at least one hydrophobic polymer block consisting essentially of hydrophobic residues adjacent at least one hydrophilic polymer block consisting essentially of hydrophilic residues.

19. The method of Claim 18, further including the steps of:  
providing a photo-affinity crosslinking agent having a photo-responsive coupling moiety and a protein coupling moiety operable to covalently bind a capture molecule, said crosslinking agent selected from the group consisting of: aryl azides, fluorinated aryl azides, and benzophenones;  
providing means for irradiating the coated optical surface with light suitable to activate the photo-responsive coupling moiety; and  
operating said irradiation means to irradiate the coated optical surface in the presence of the crosslinking agent to activate the photo-responsive coupling moiety, thereby covalently coupling the crosslinking agent to the coating.

20. The method of Claims 18 or 19, further including the steps of:  
providing one or more species of capture molecules, each said one or more species being constructed to specifically bind a corresponding analyte and each species including a thiol group reactively disposed therein; and  
coupling said capture molecules to said coating via said thiol group.

21. The method of Claim 19, further including the steps of:  
providing one or more species of capture molecules, each said one or more species being constructed to specifically bind a corresponding analyte; and  
coupling the capture molecules to the protein coupling moiety of the crosslinking agent.

22. The method of Claim 21, wherein a plurality of species of capture molecules are provided; wherein said step of providing means for irradiating includes a step of providing means to separately sequentially irradiate a plurality of portions of the coated region of the waveguide; and wherein said step of irradiating is a step of selectively

sequentially irradiating said portions of the coated region in the presence of the crosslinking agent.

23. The method of Claims 21 or 22, wherein said step of coupling said capture  
5 molecules to said crosslinking agent is performed prior to said step of irradiating.

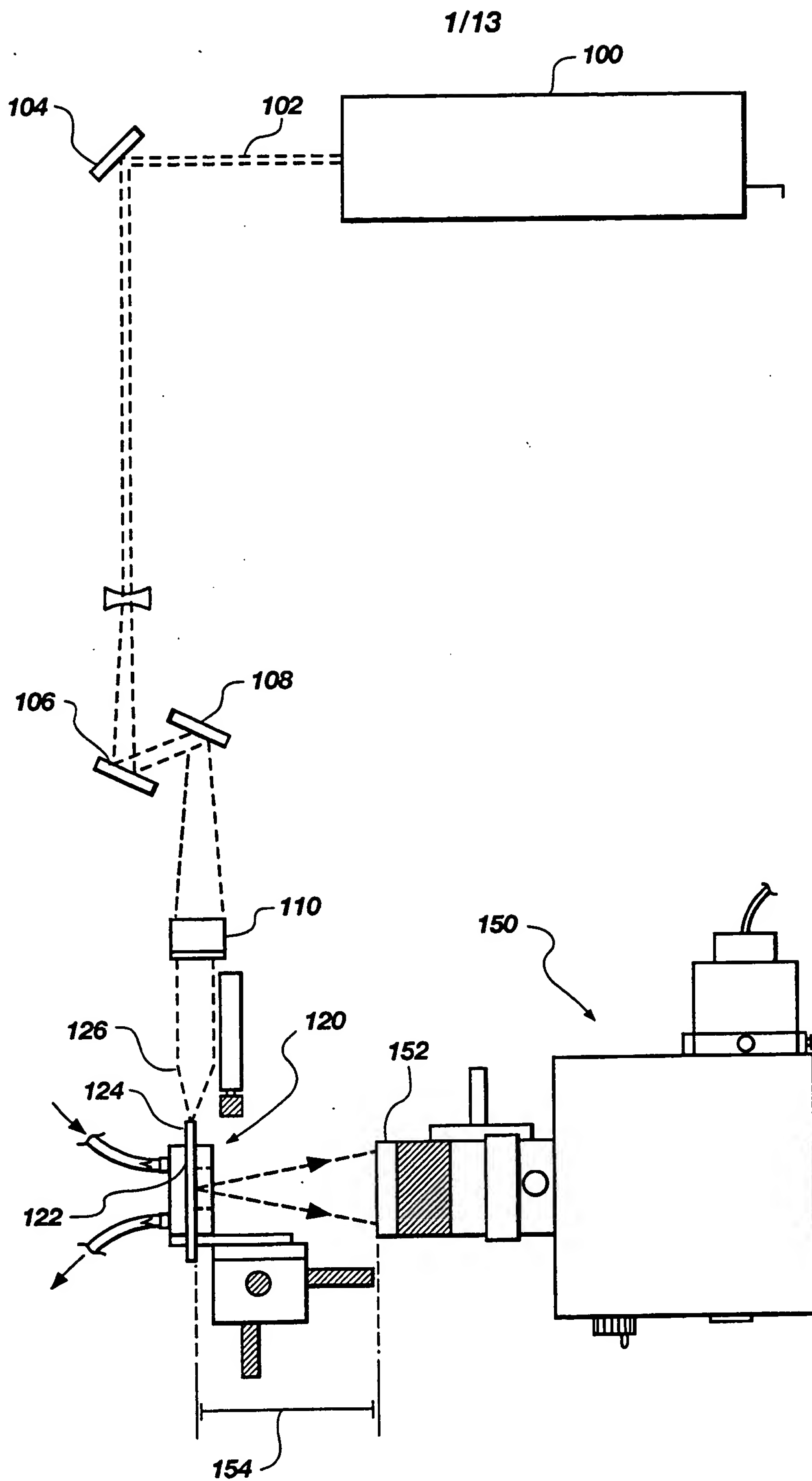
24. The method of Claims 22 or 23, wherein said step of separately sequentially  
irradiating includes the steps of:  
irradiating one of the portions of the coated region in the presence of free crosslinker;  
10 coupling one of the species of capture molecules to the crosslinking agent; and  
repetitively alternating said steps of irradiating a portion and coupling a species of  
capture molecule, said step of repetitive alternation being performed with different  
species of said plurality of species for each of the portions.

15 25. A method of performing a solid-state fluoroimmunoassay in which a  
capture molecule specific for an analyte is coupled to an optical substrate, and in which  
binding of the analyte to the capture molecule results in a fluorescent signal  
corresponding to the presence of the analyte in a sample, comprising the steps of:  
providing a biosensor defined as in Claim 1;  
20 providing a light source disposed to deliver a light beam into the waveguide through the  
semi-cylindrical lens;  
providing detection means disposed for detecting fluorescent light which impinges on a  
plane parallel to and displaced from said waveguide surfaces;  
contacting the waveguide surface having the capture molecules immobilized thereon with  
25 a solution comprising a buffer, a plurality of tracer molecules capable of binding  
to the capture molecule in the presence of the analyte and of emitting a  
fluorescent signal upon stimulation with the light beam, and an unknown amount  
of analyte molecules;  
incubating the waveguide surface with the contacting solution to permit binding of the  
30 tracer molecules to the analyte molecules and the capture molecules;  
operating the light source to produce evanescent light from the waveguide to stimulate  
the tracer molecules;  
operating the detection means to detect the fluorescent signals emitted by the tracer  
molecules; and

analyzing the fluorescent signals emitted to determine the amount of the analyte in the test sample.

26. The immunoassay method of Claim 25, wherein in the step of providing a  
5 waveguide biosensor, the waveguide has an optical surface according to Claims 10, 11 or 12, and wherein the provided detection means includes a plurality of photodetector elements and lens means oriented to direct light from a plurality of portions of the coated region onto respective corresponding subsets of the photodetector elements.

10 27. The immunoassay method of Claims 25 or 26, wherein the method is performed without a wash step for washing unbound tracer from the surface.

**Fig. 1**



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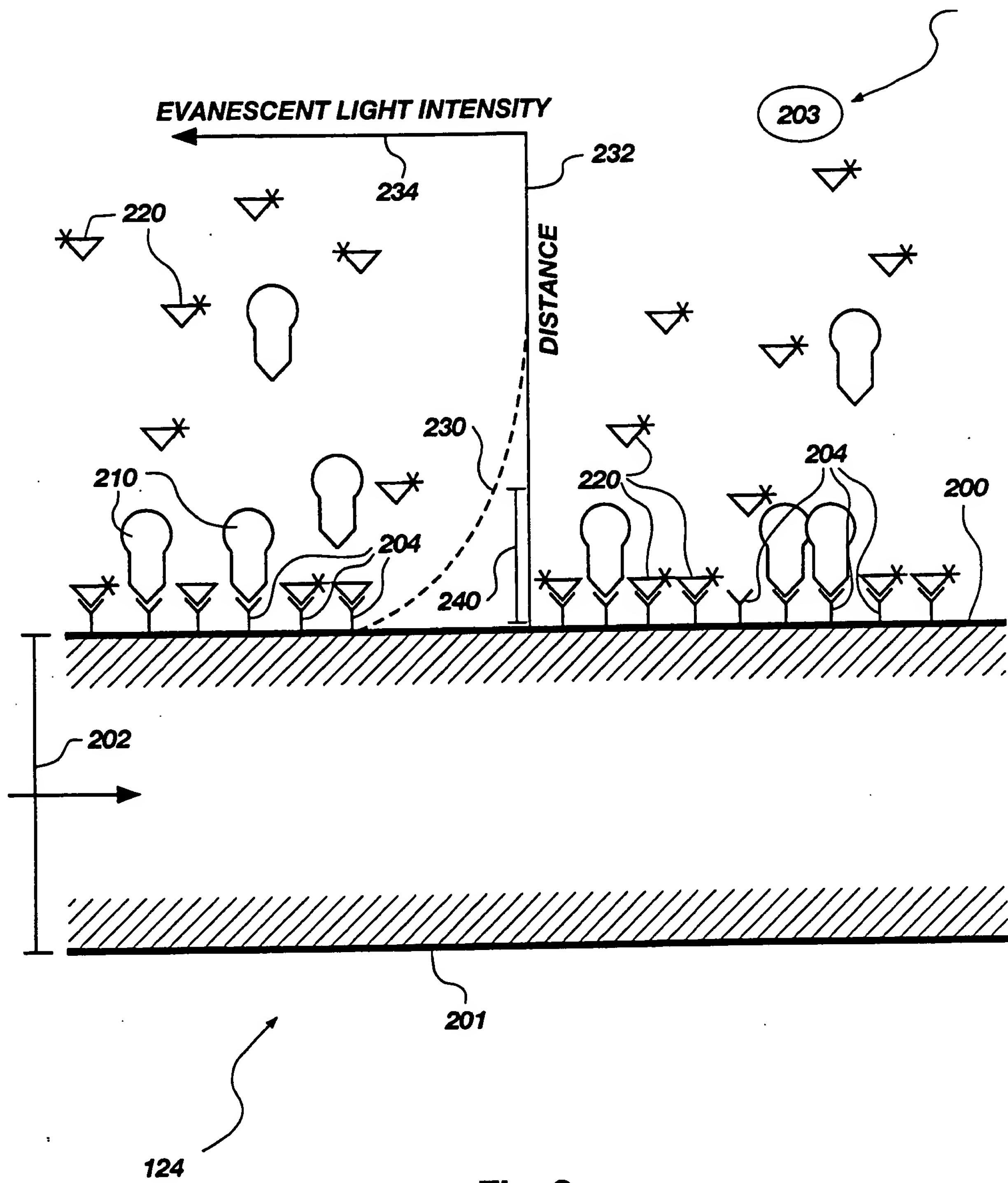
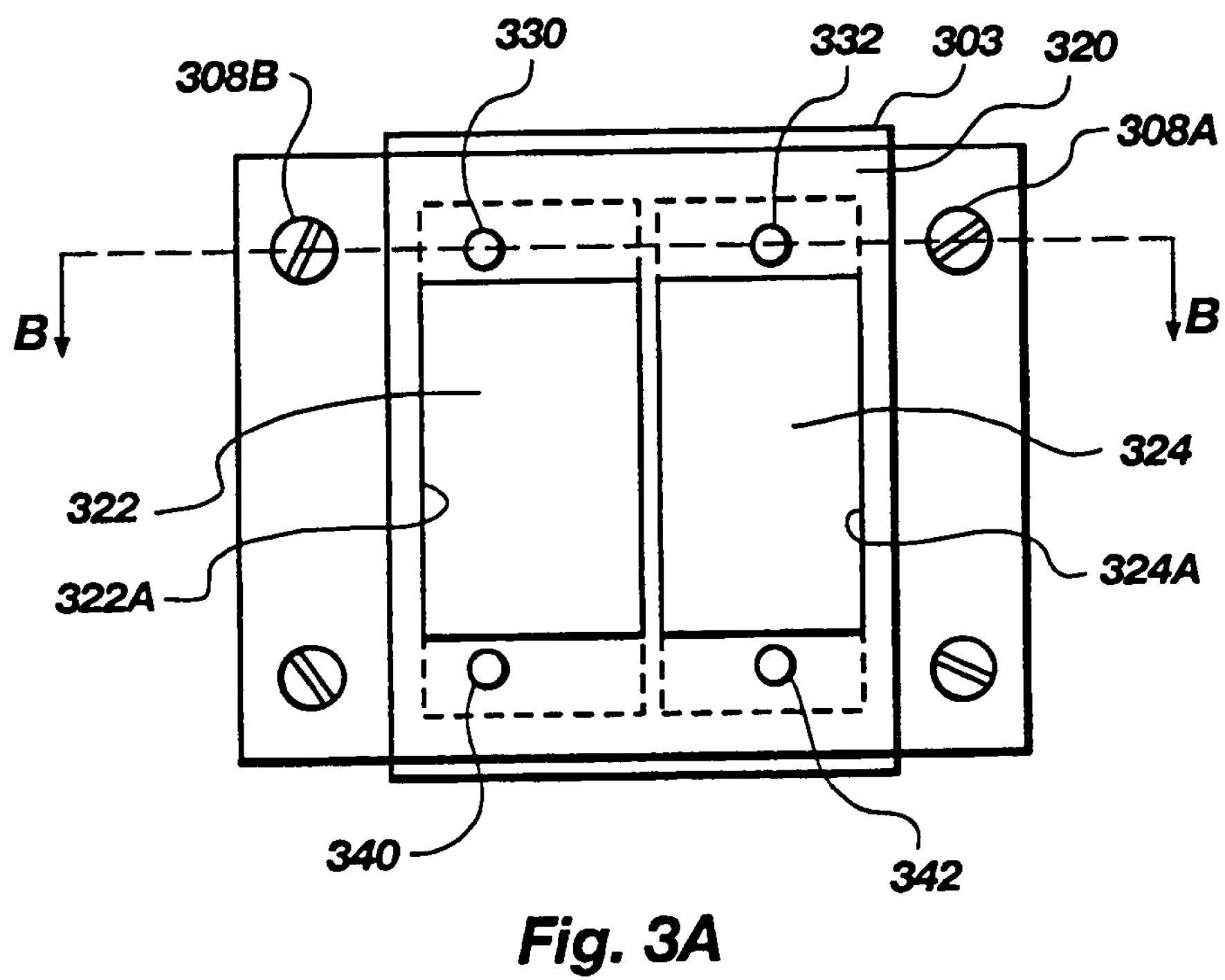
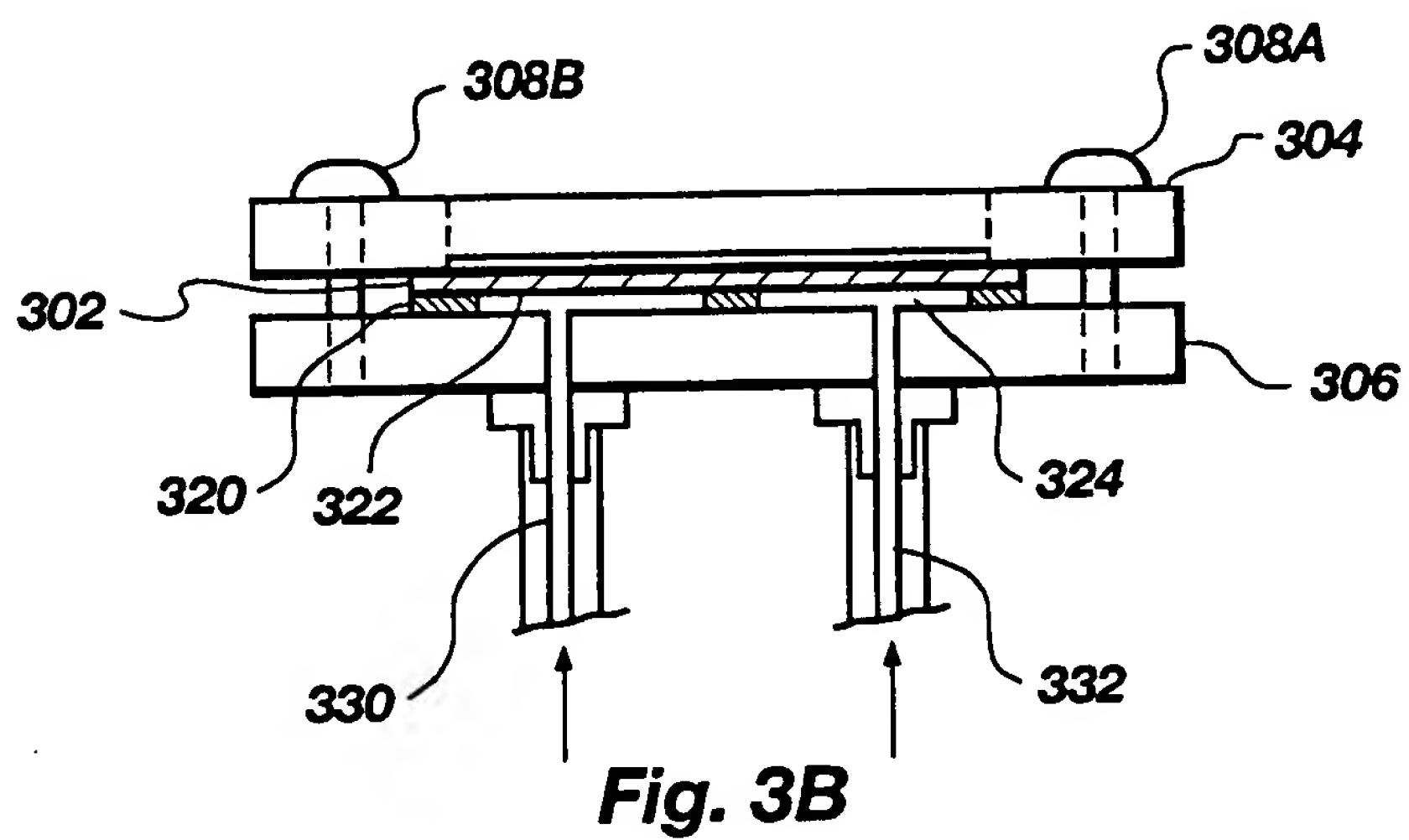
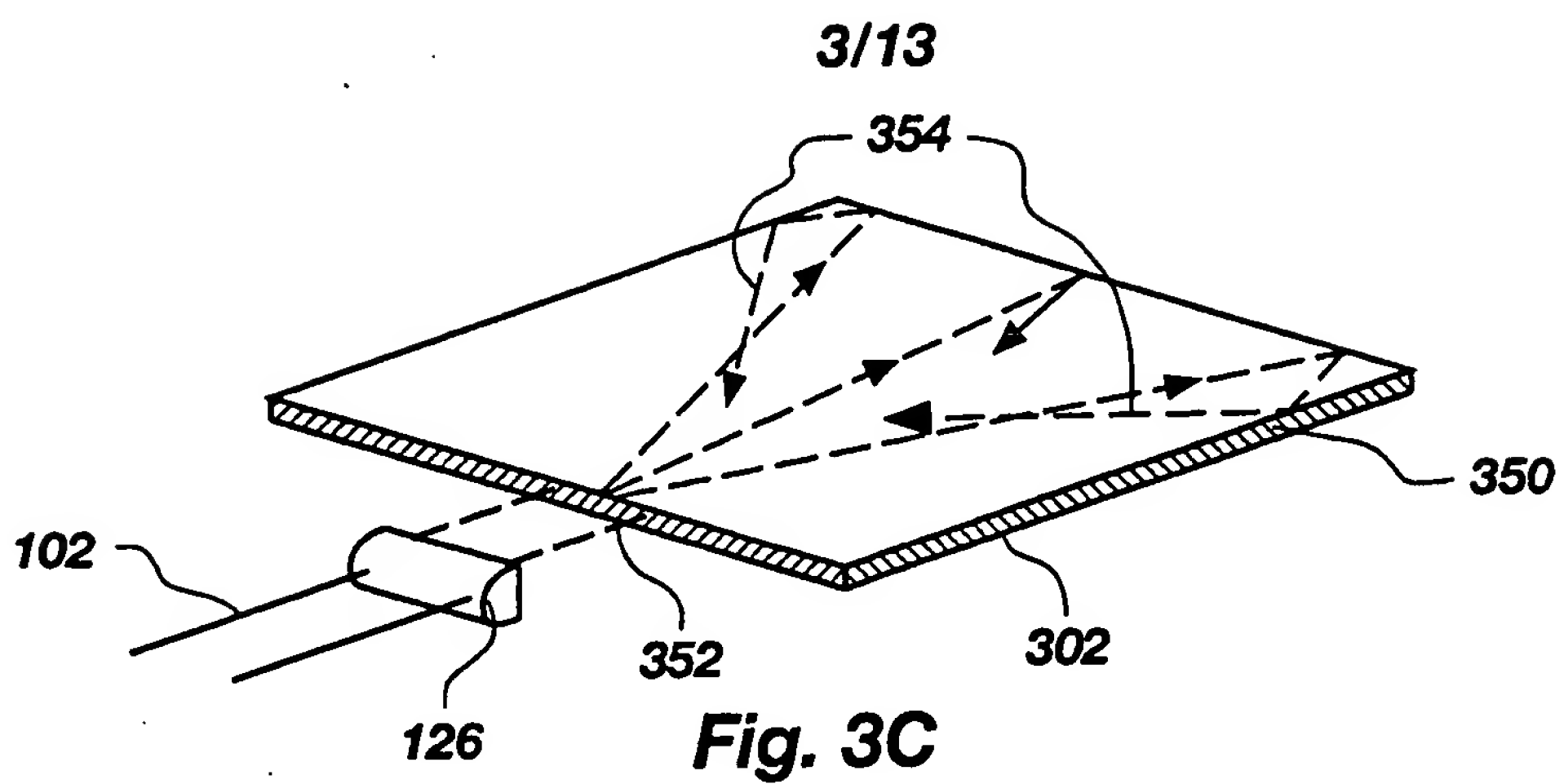
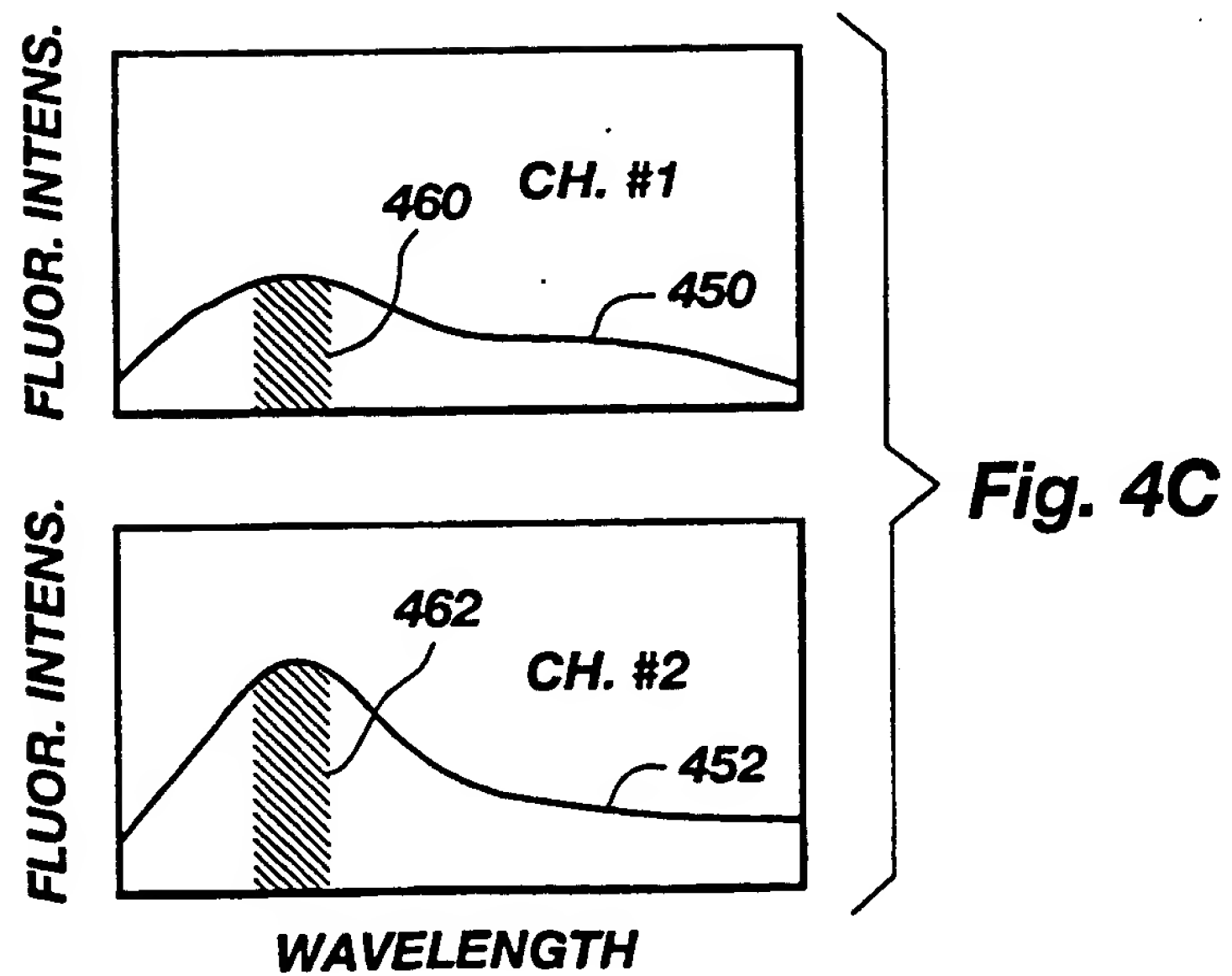
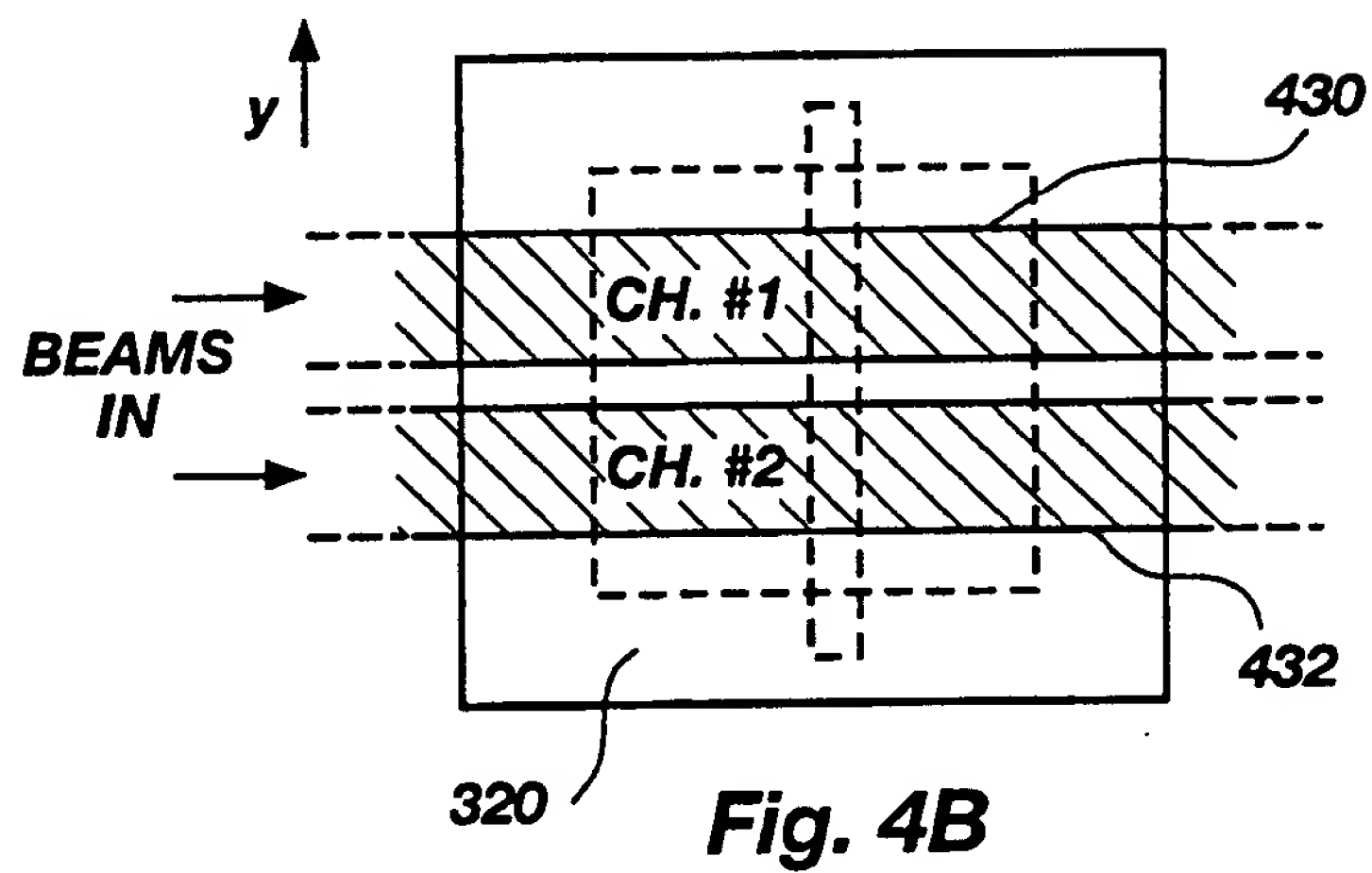
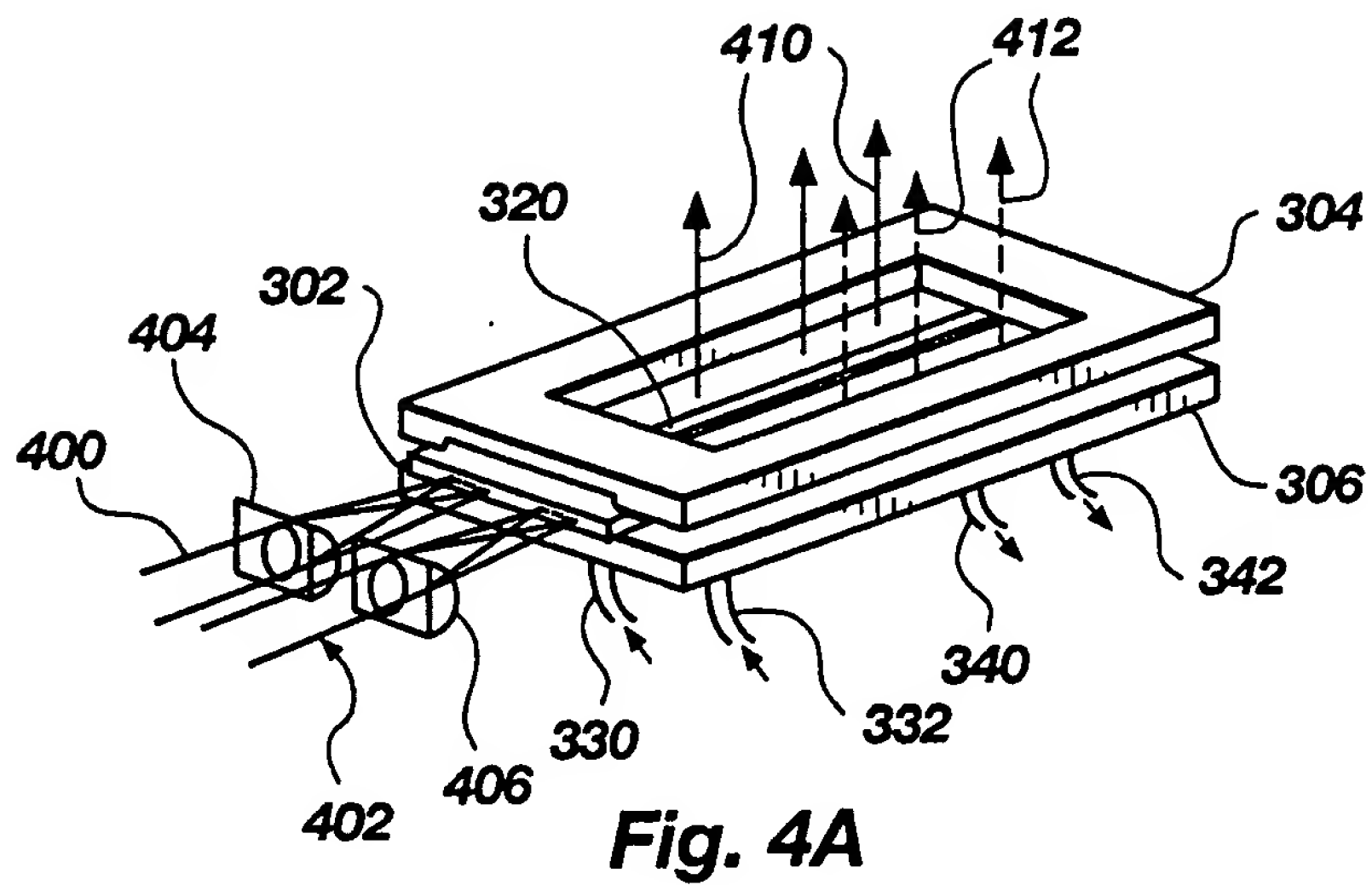


Fig. 2



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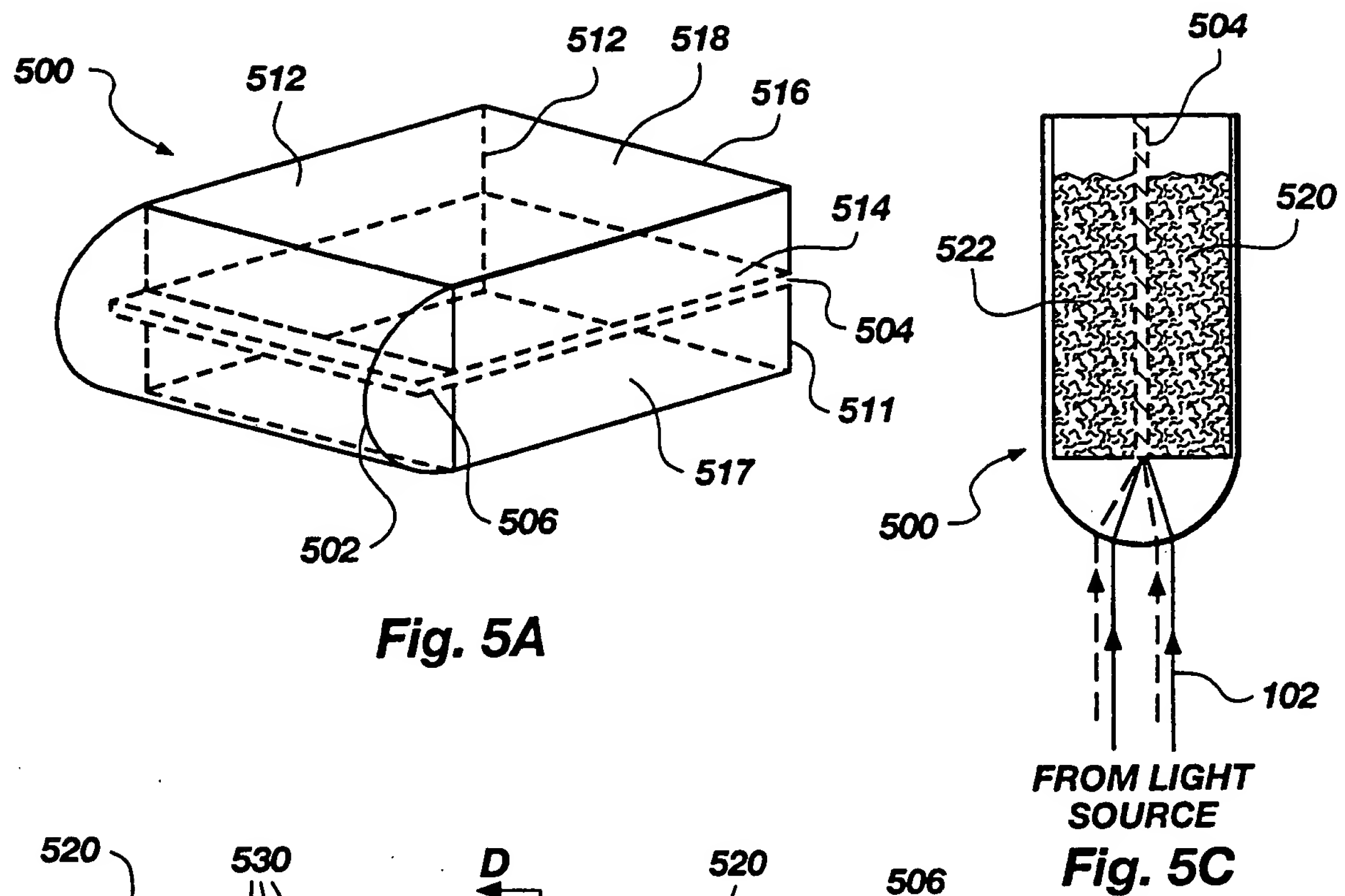


Fig. 5A

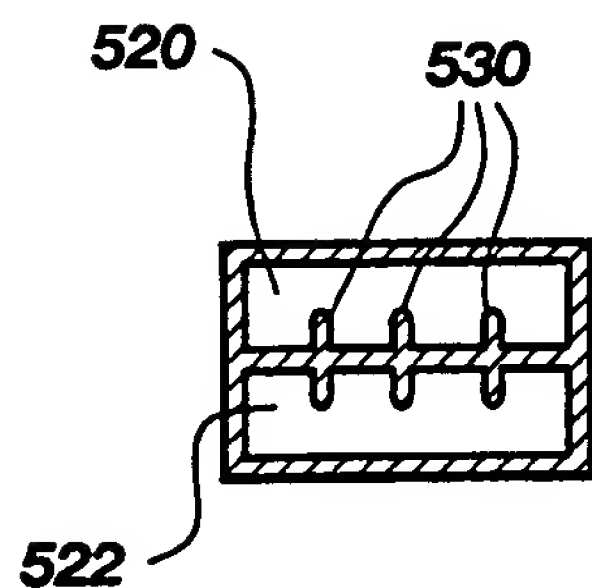
FROM LIGHT  
SOURCE  
Fig. 5C

Fig. 5D

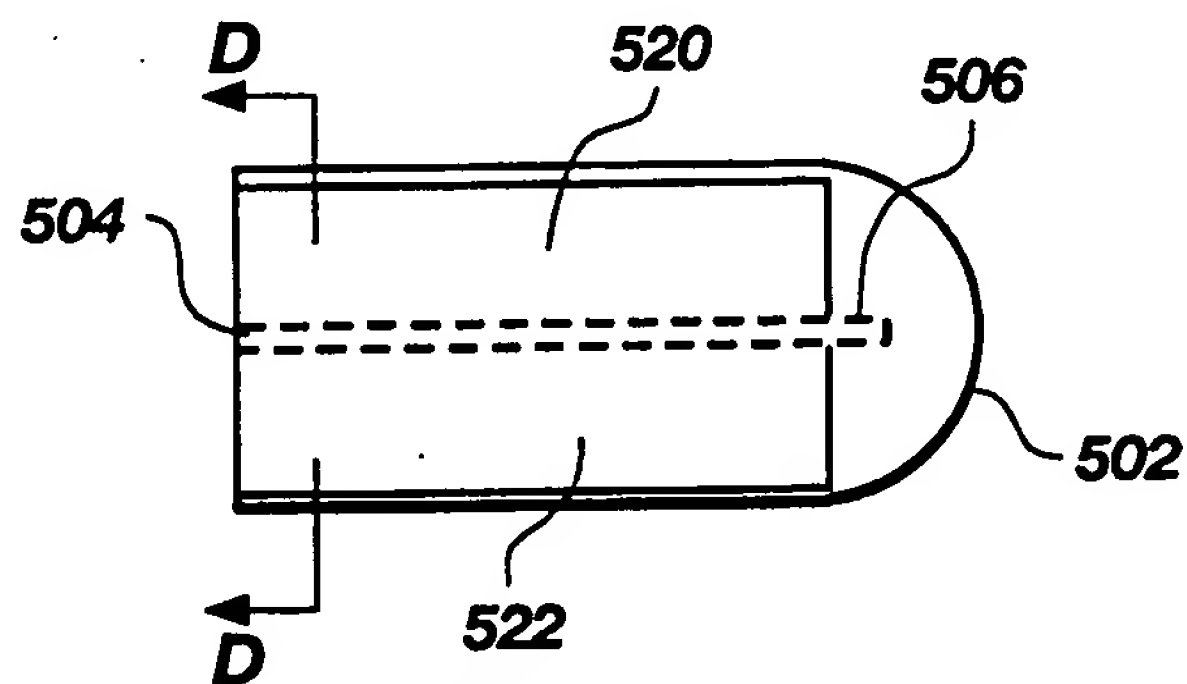


Fig. 5B

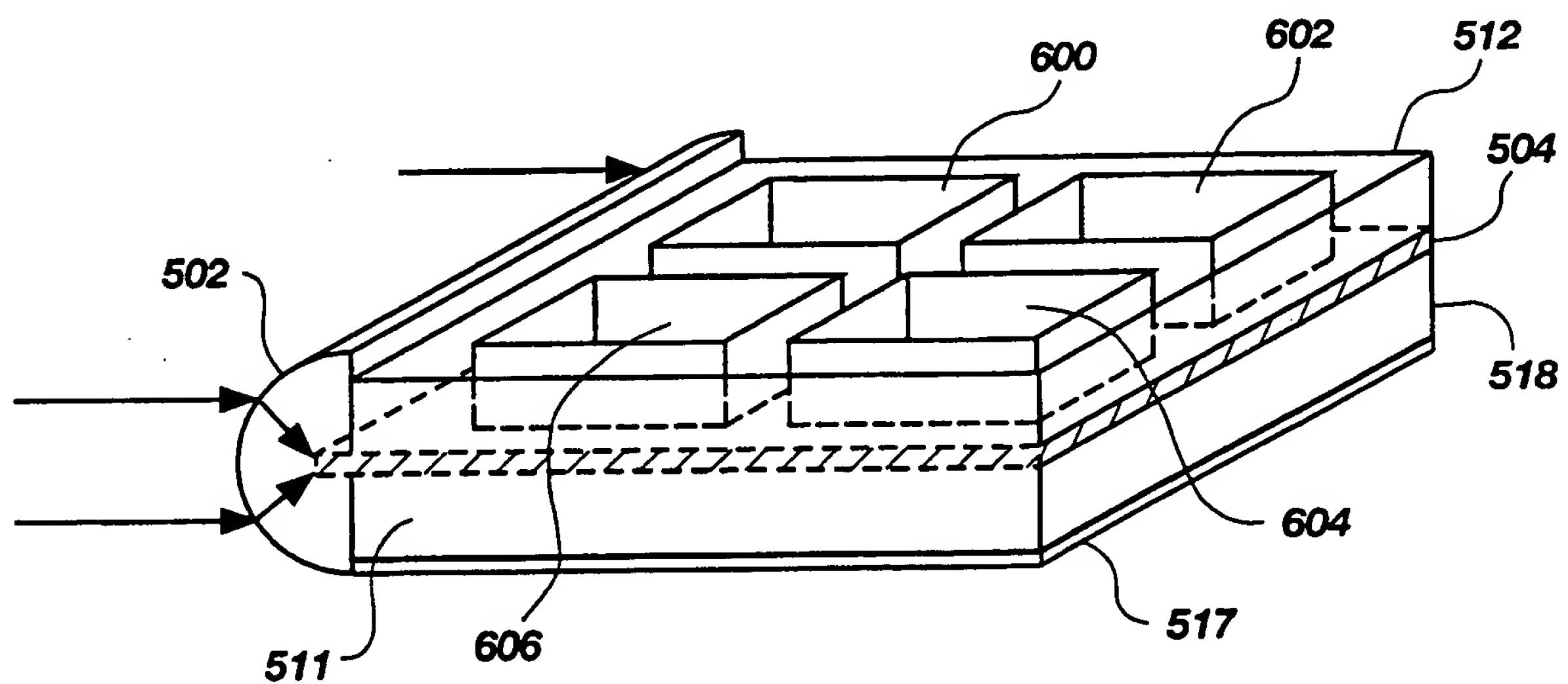


Fig. 6

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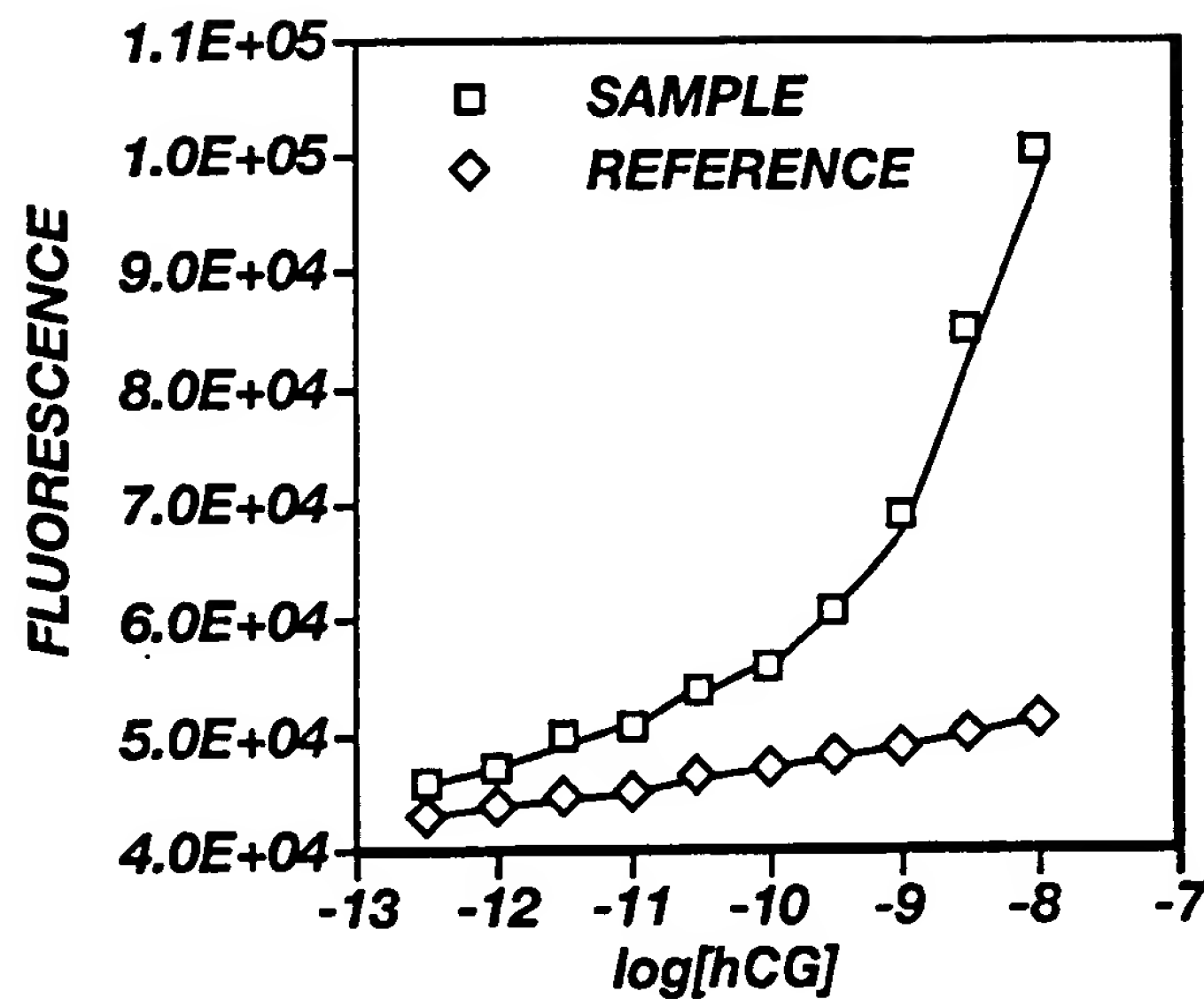


Fig. 7A

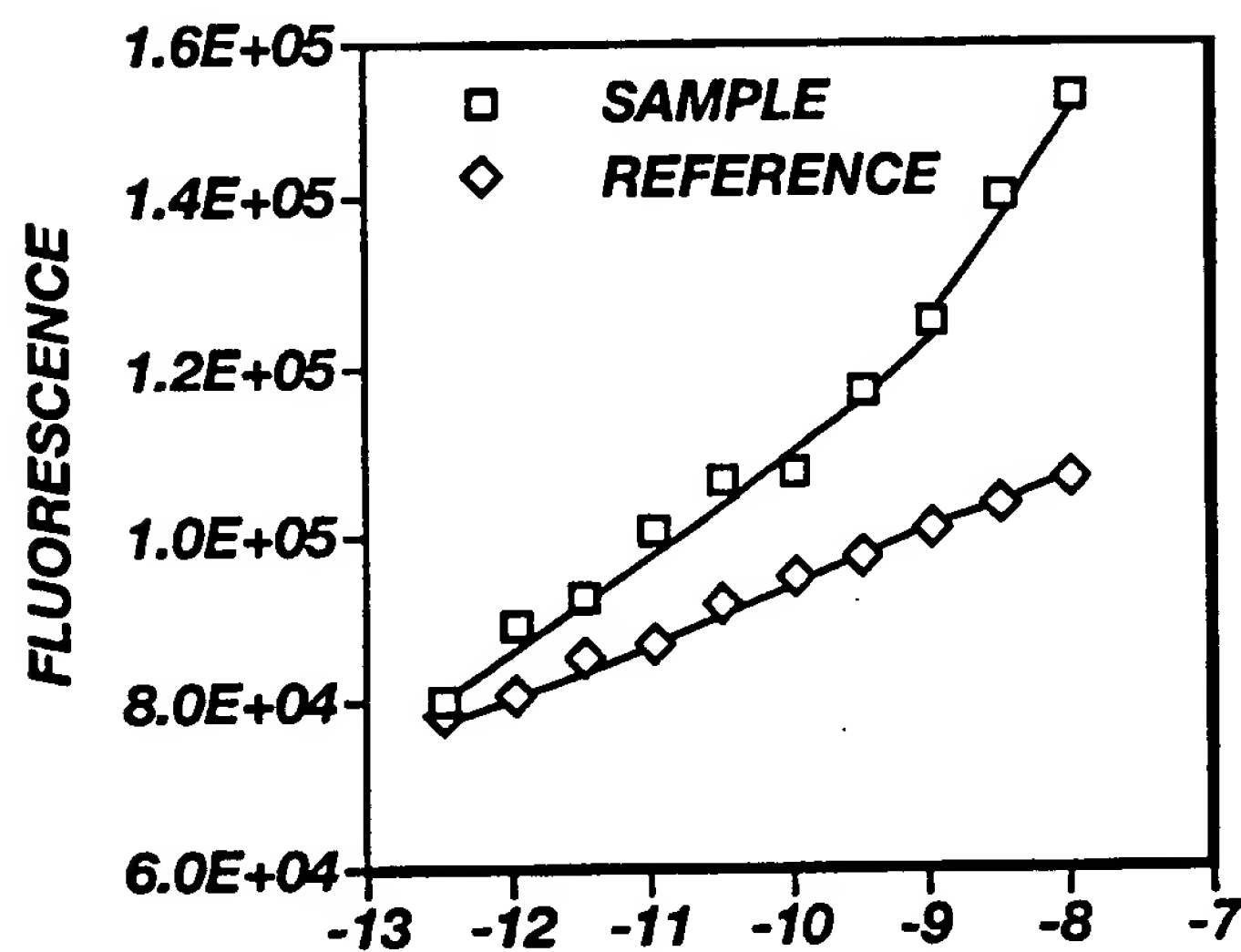


Fig. 7B

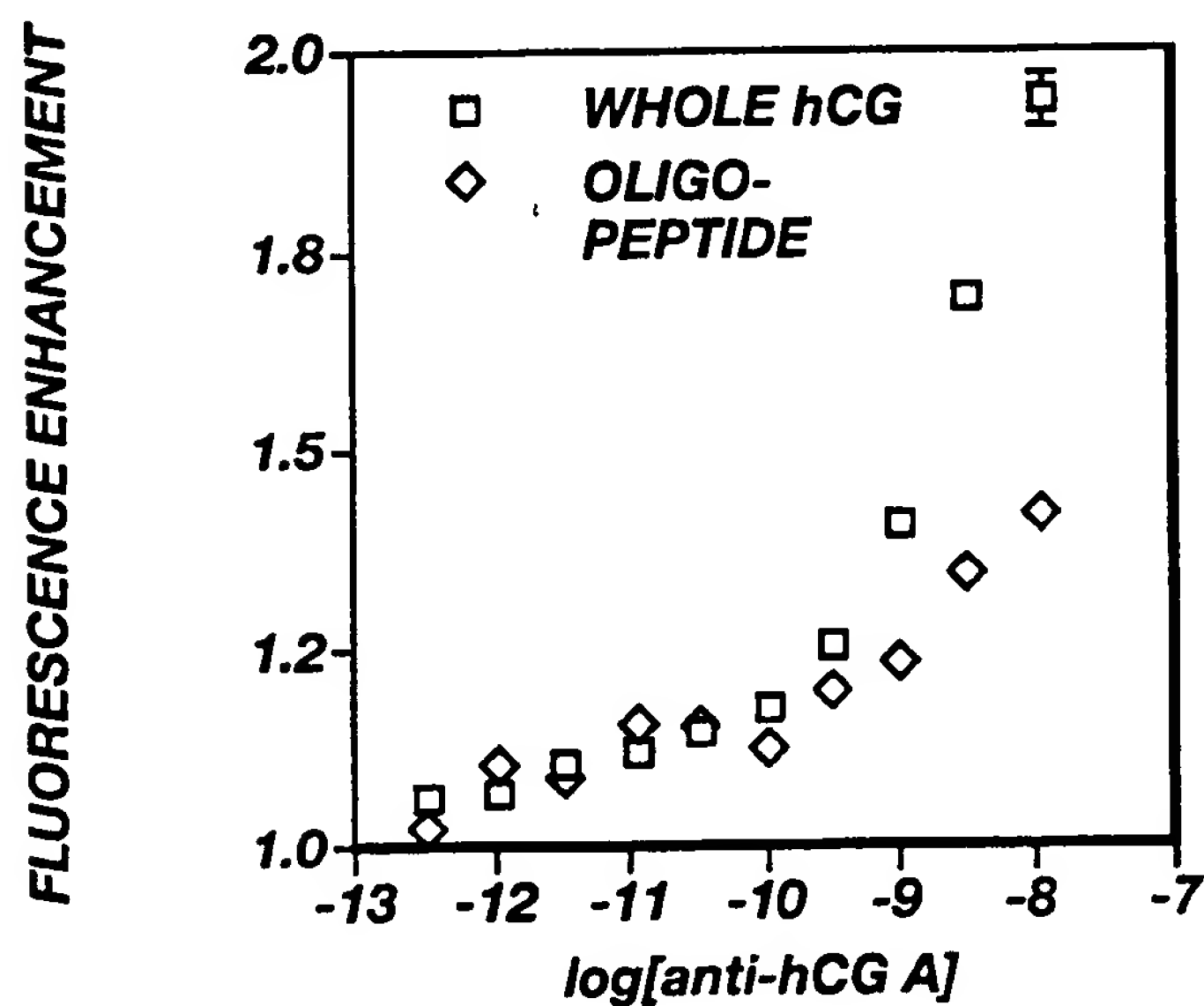


Fig. 8



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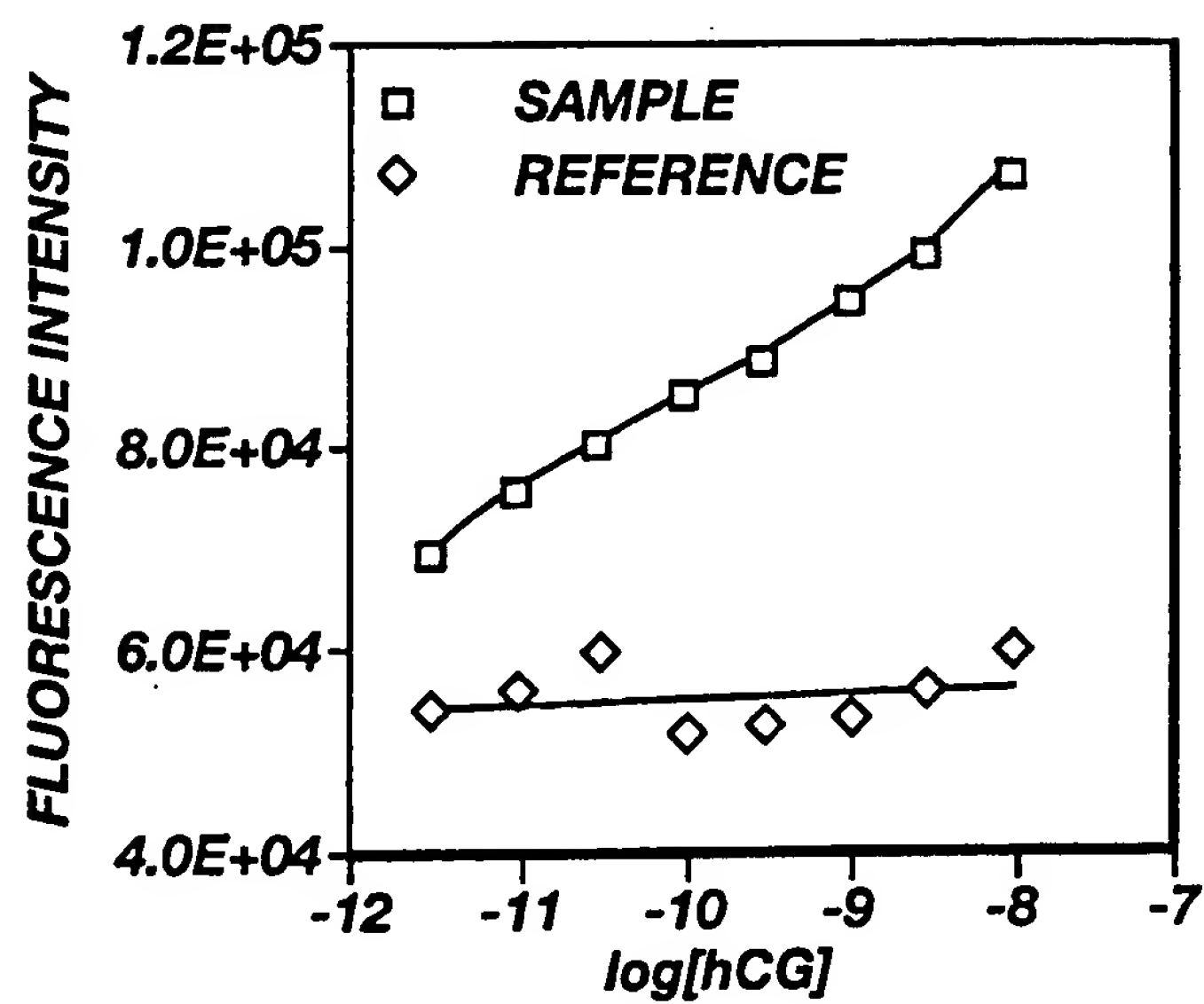


Fig. 9A

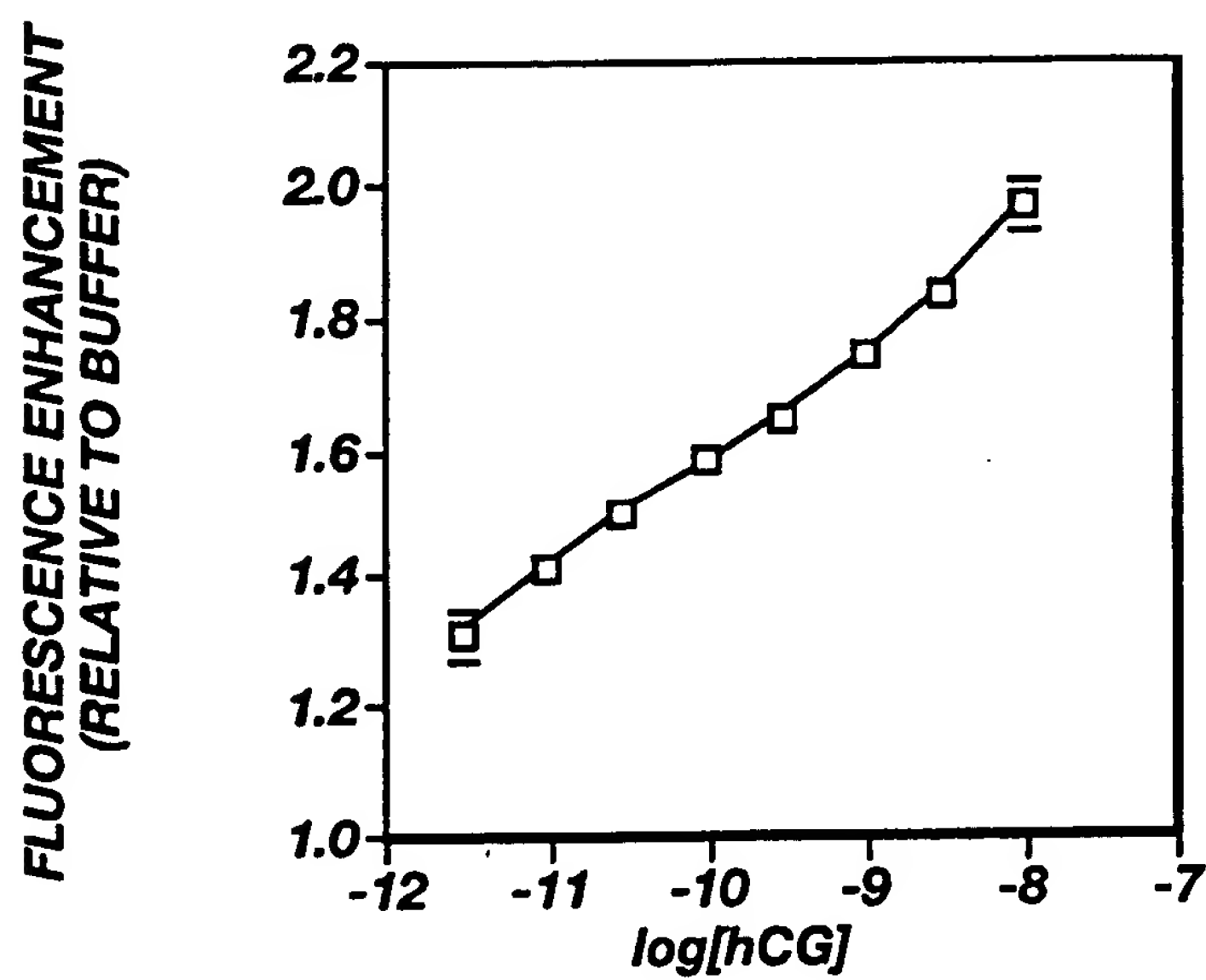


Fig. 9B

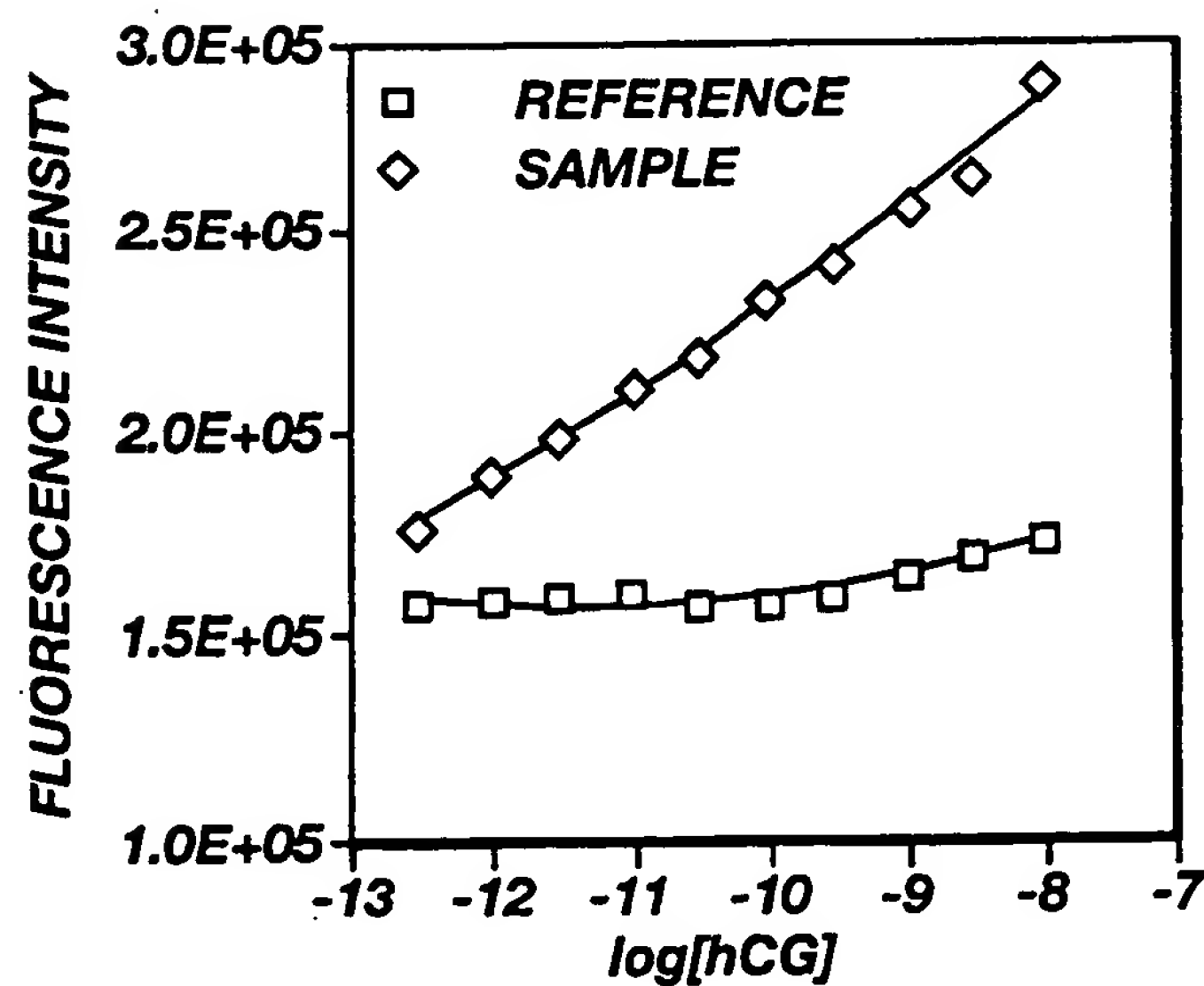


Fig. 9C

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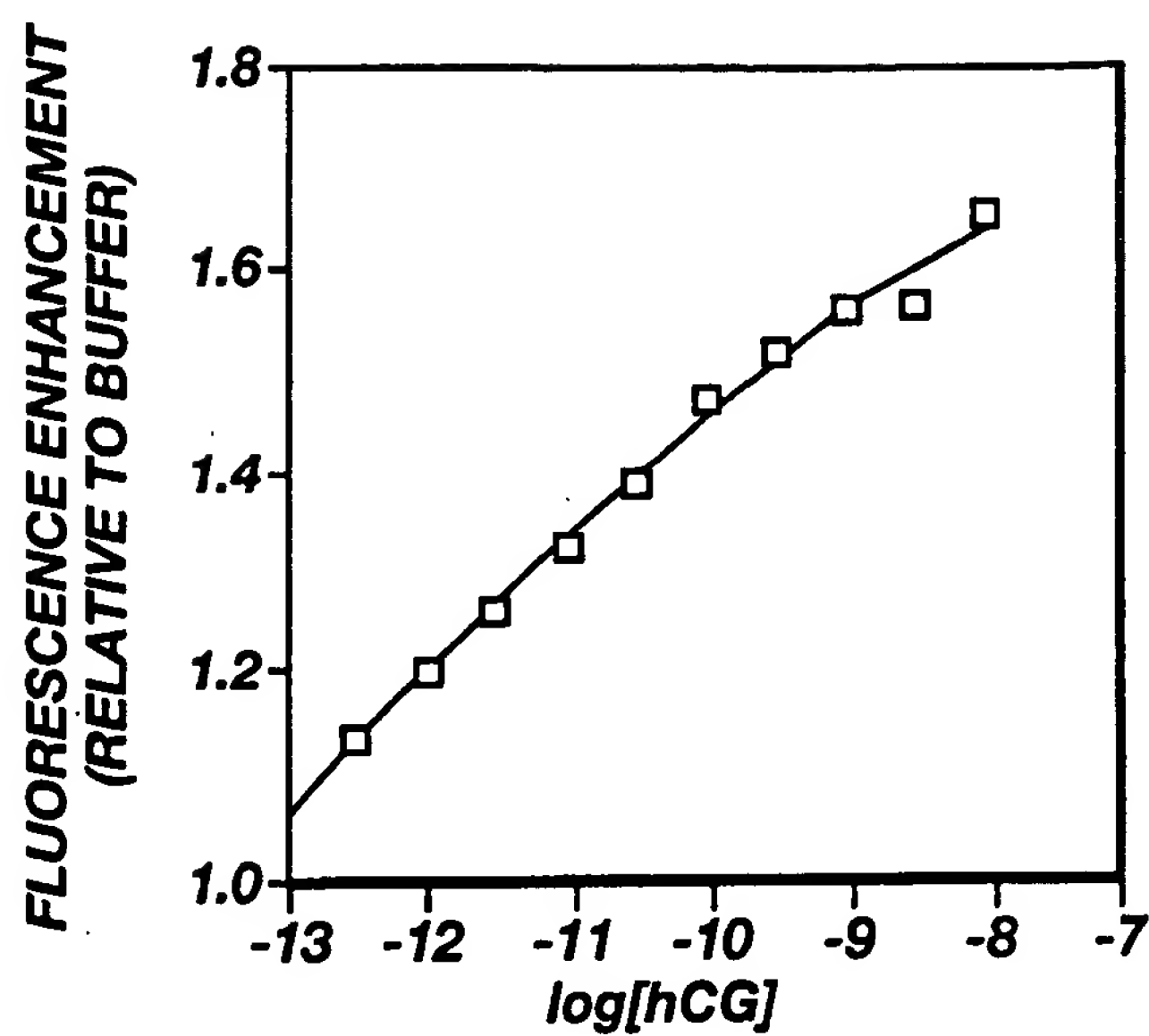


Fig. 9D

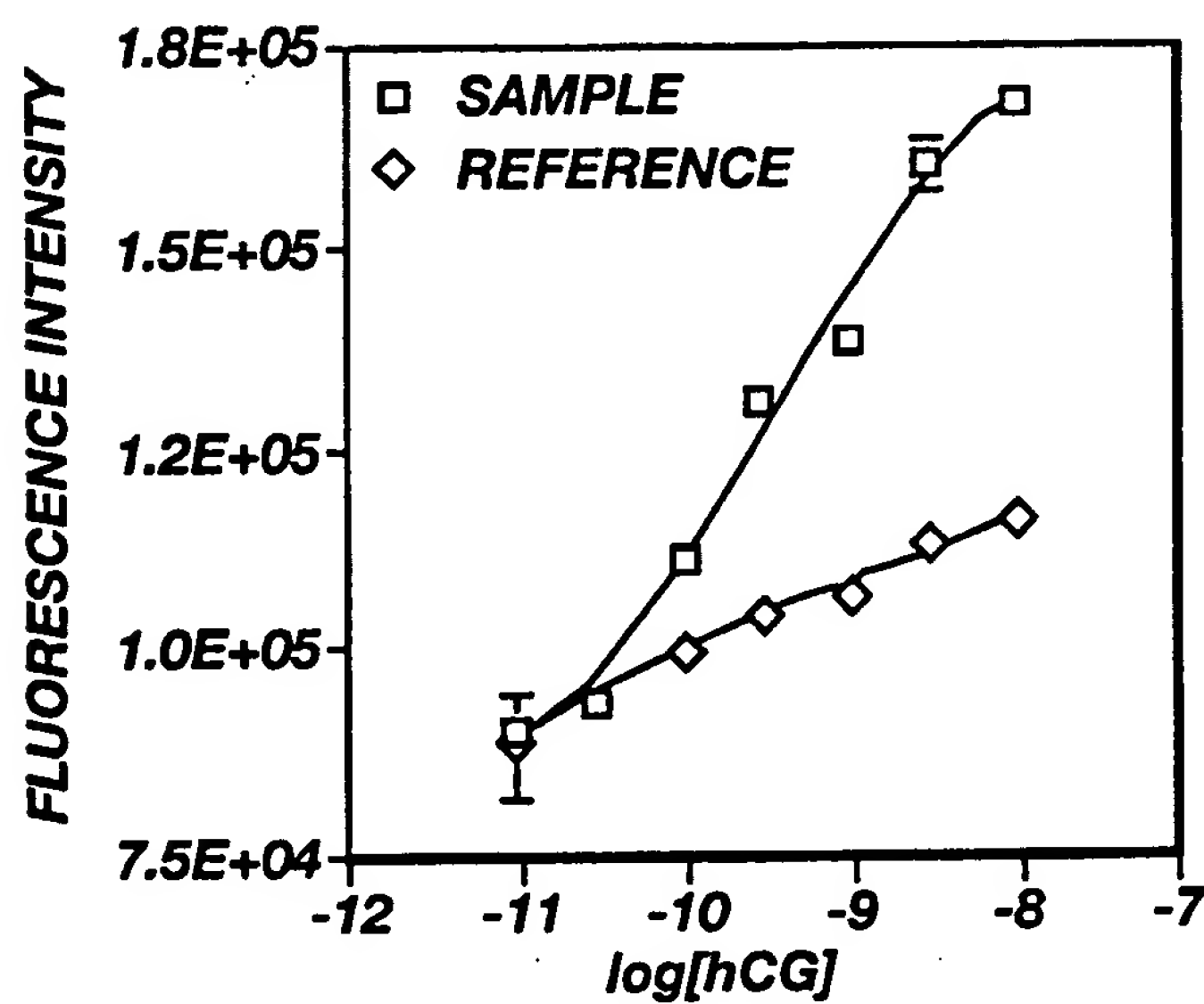


Fig. 9E

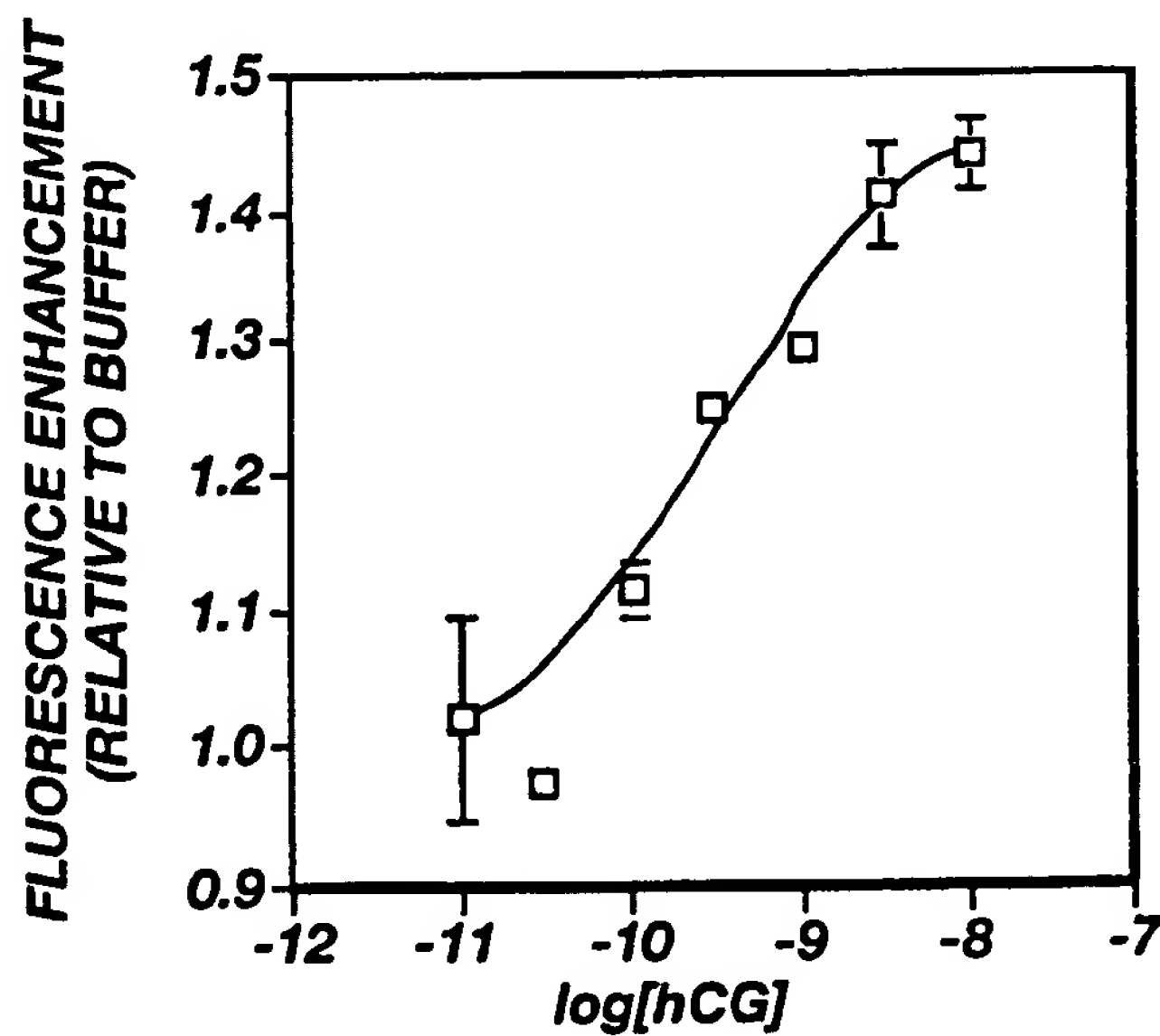
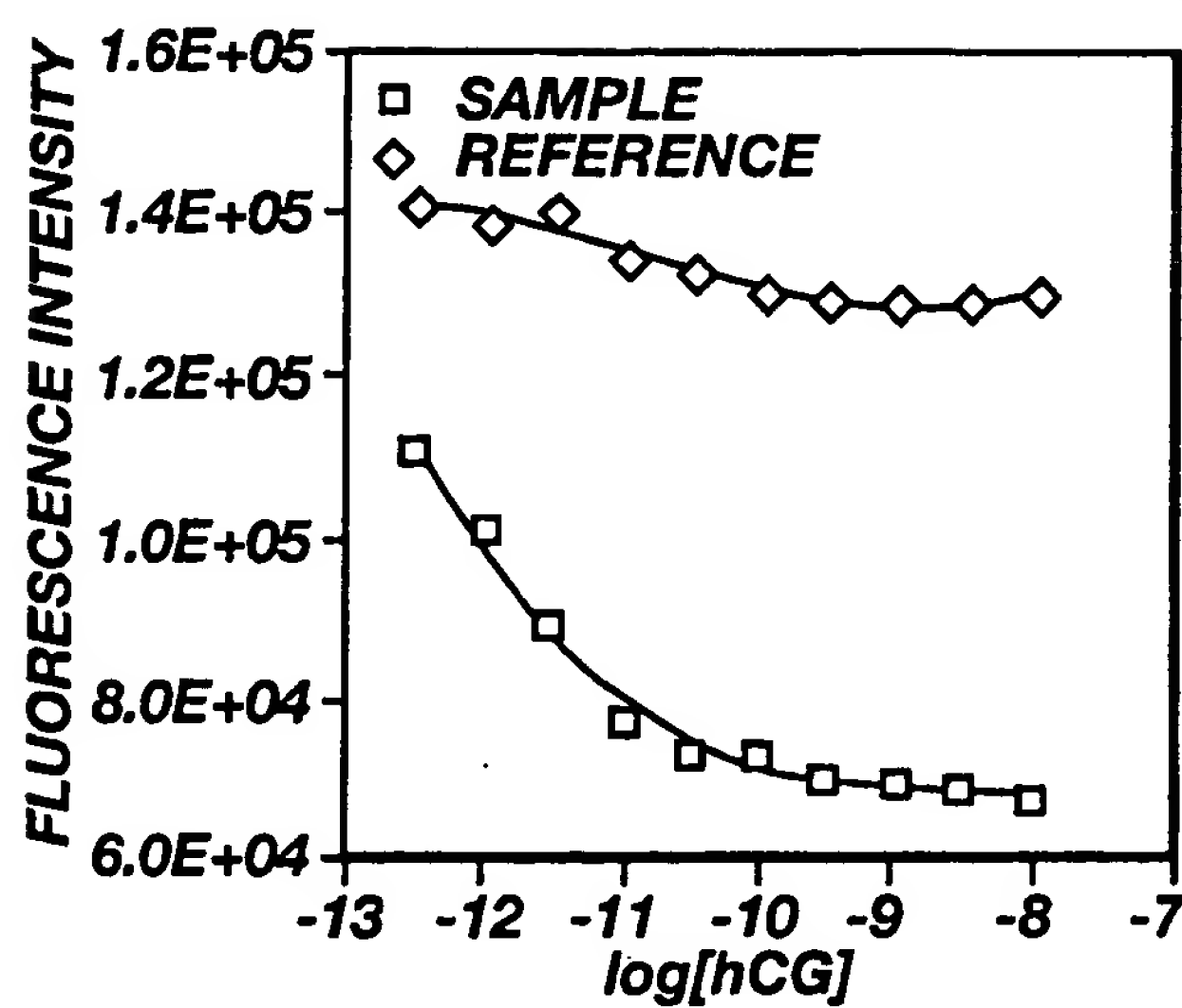
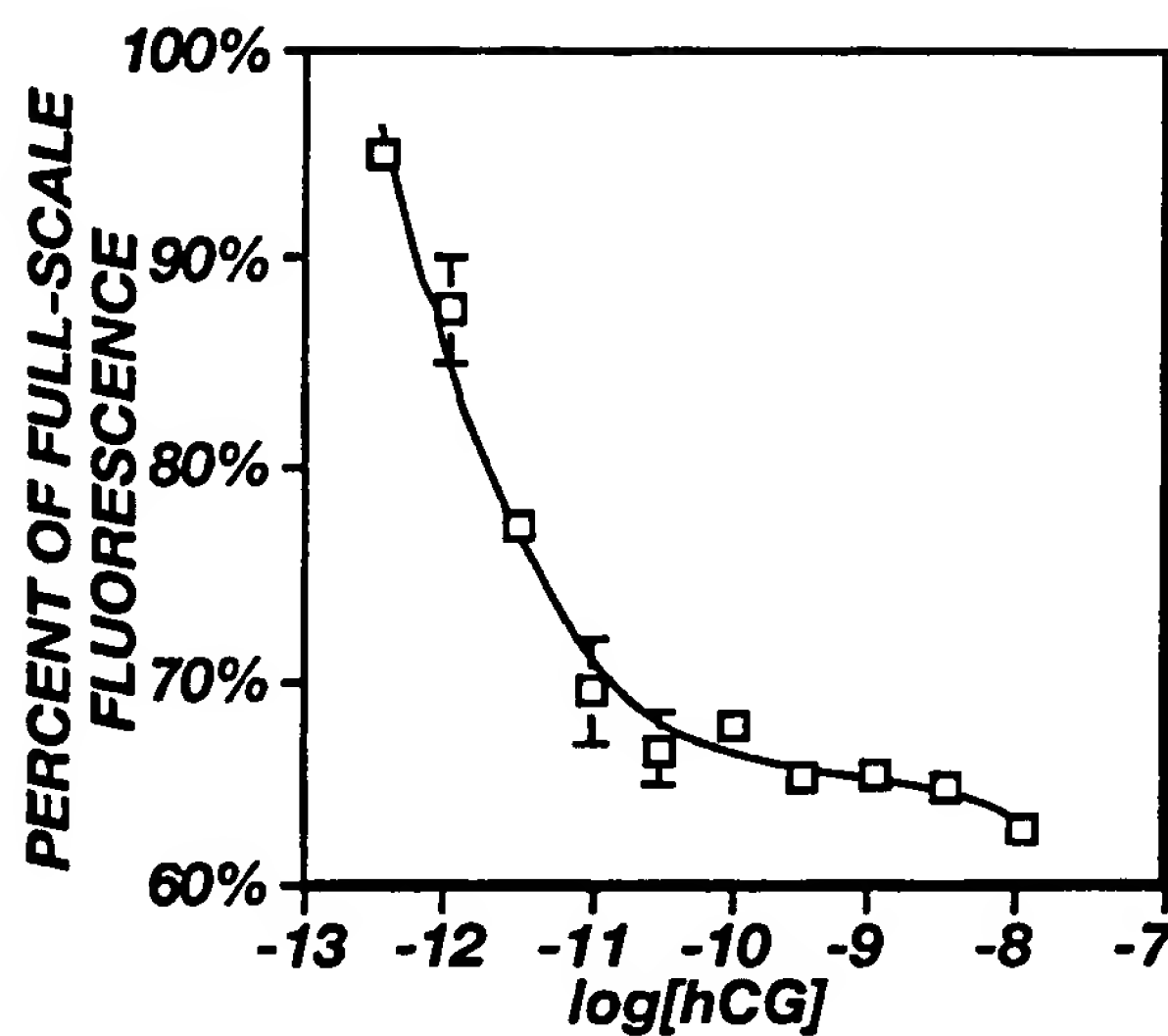
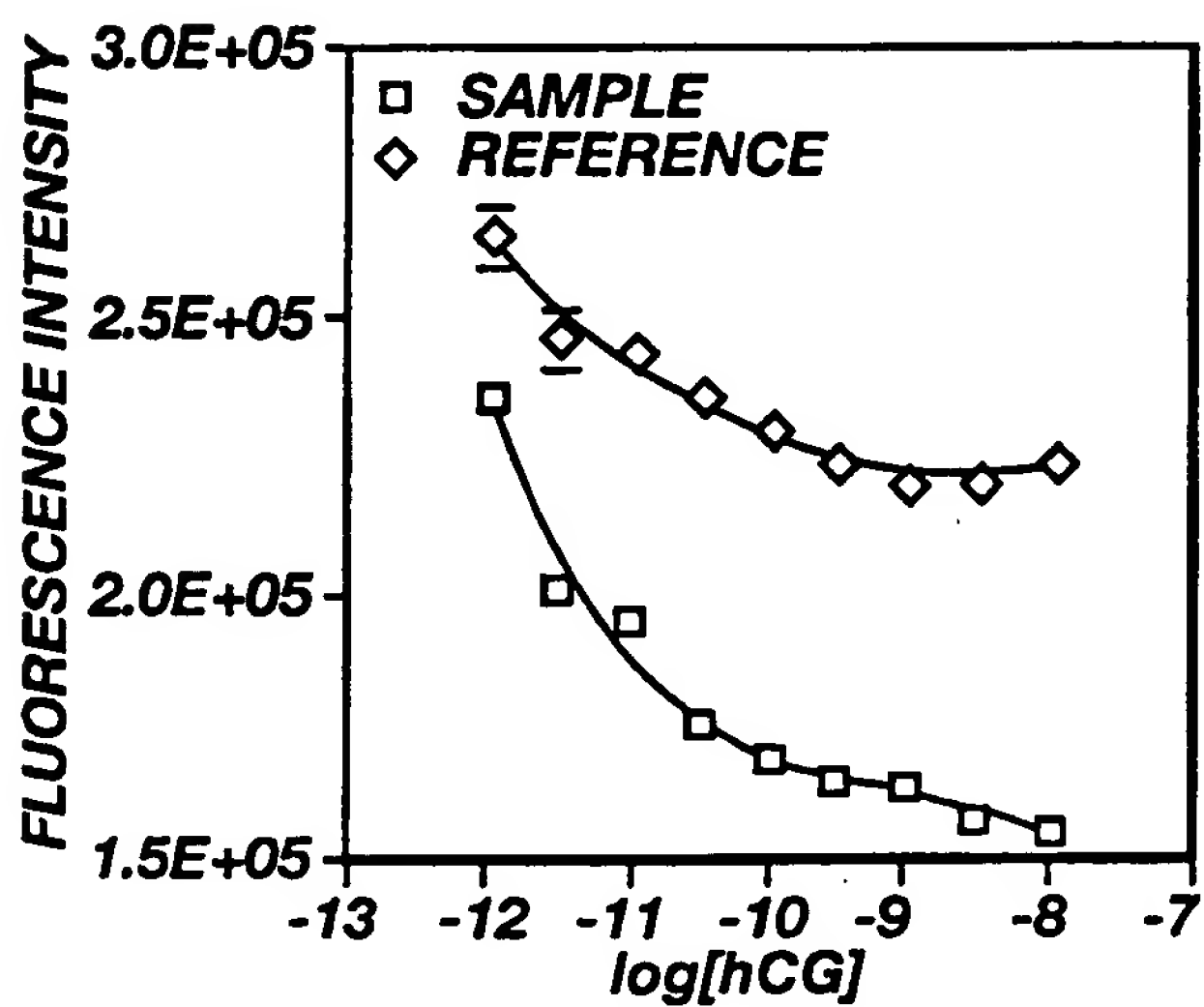
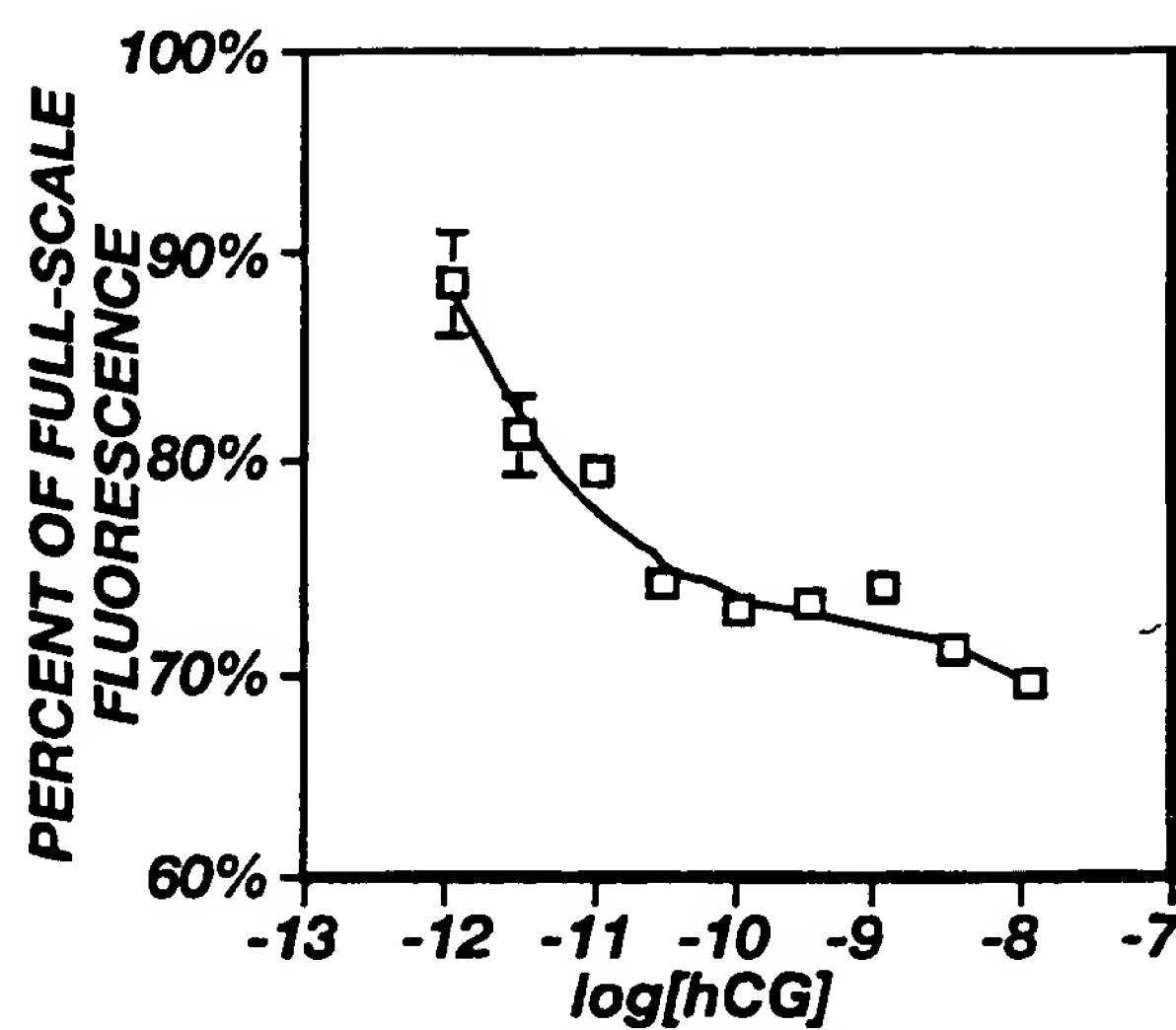
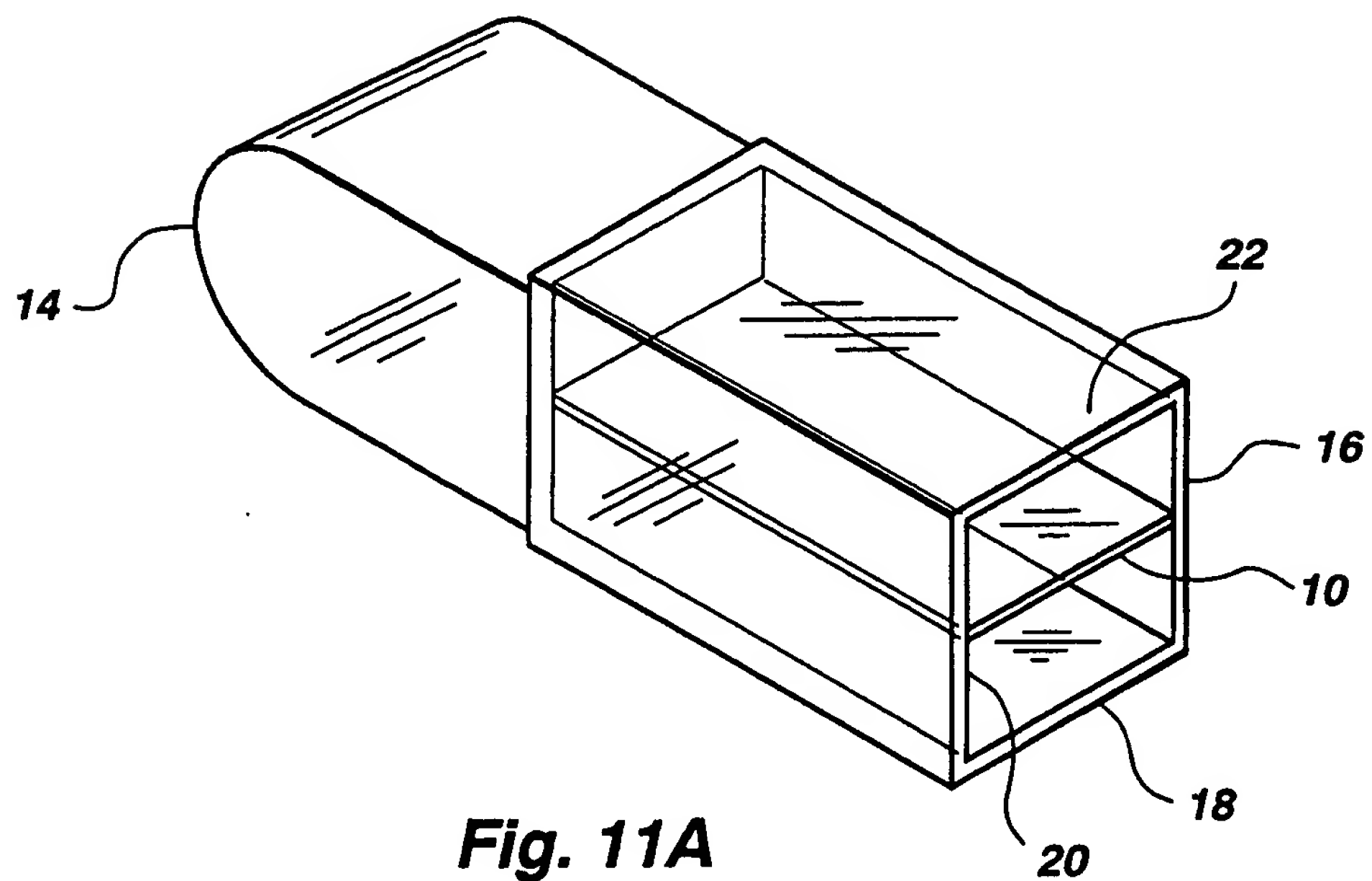
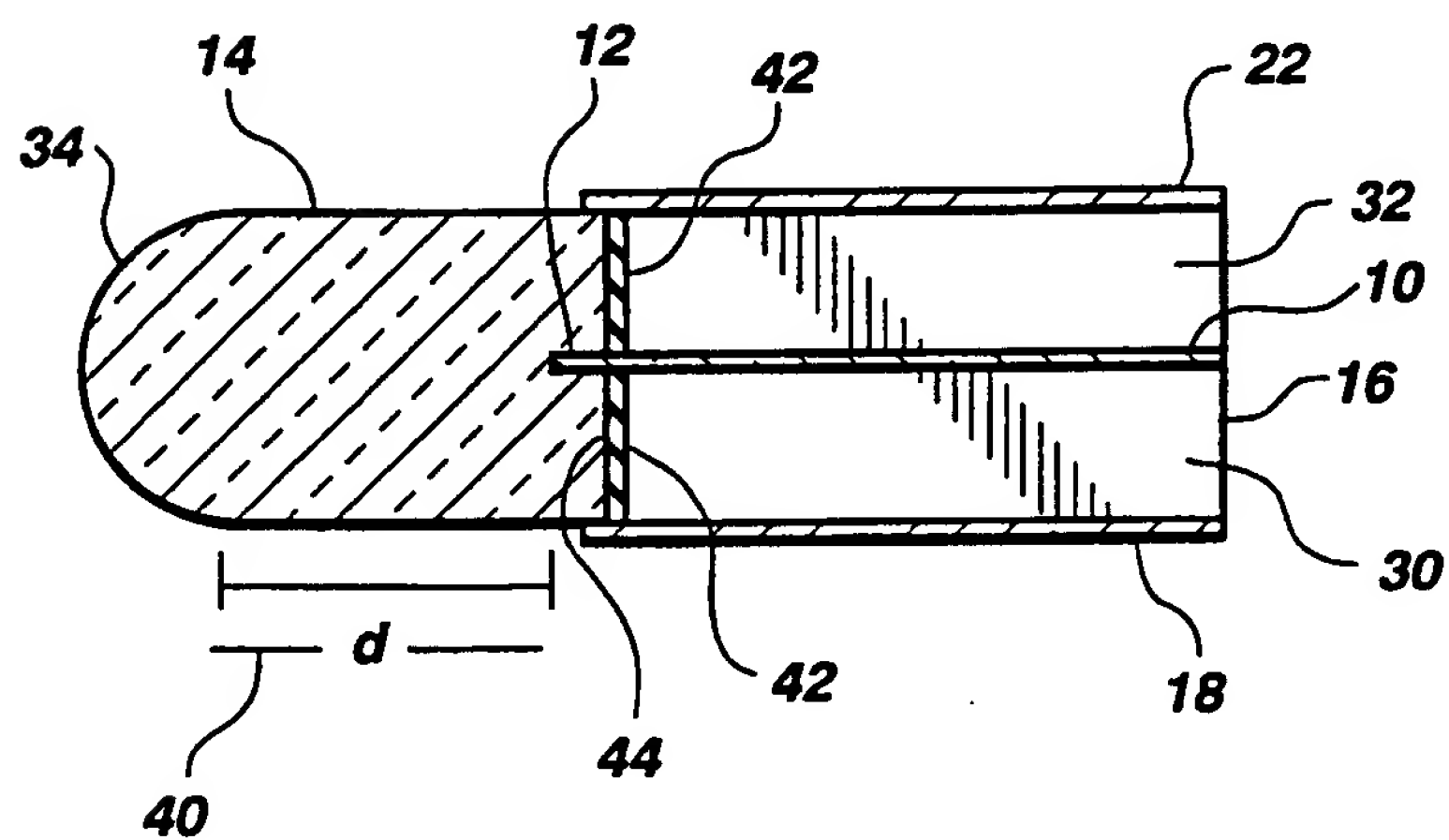
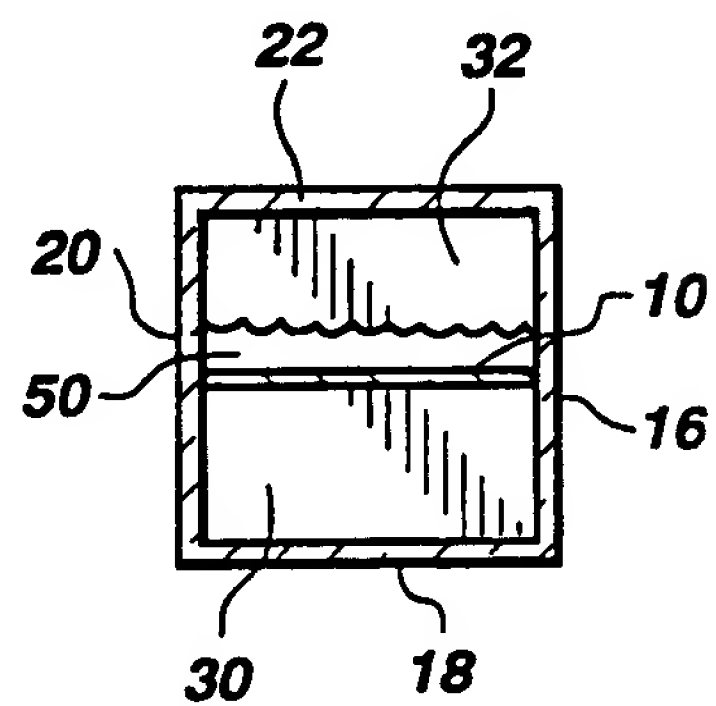


Fig. 9F

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**Fig. 10A****Fig. 10B****Fig. 10C****Fig. 10D**

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**Fig. 11A****Fig. 11B****Fig. 11C**

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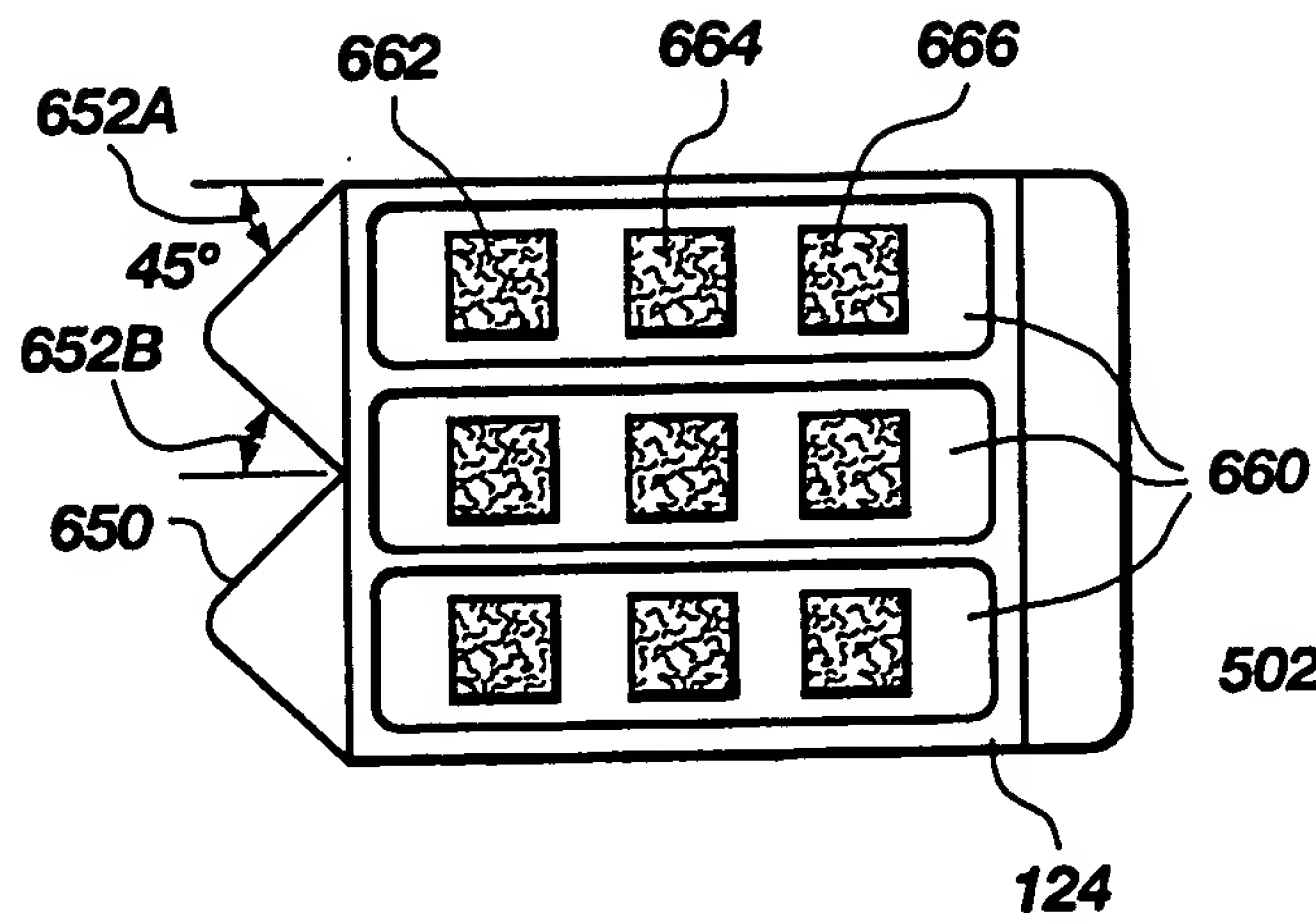


Fig. 12A

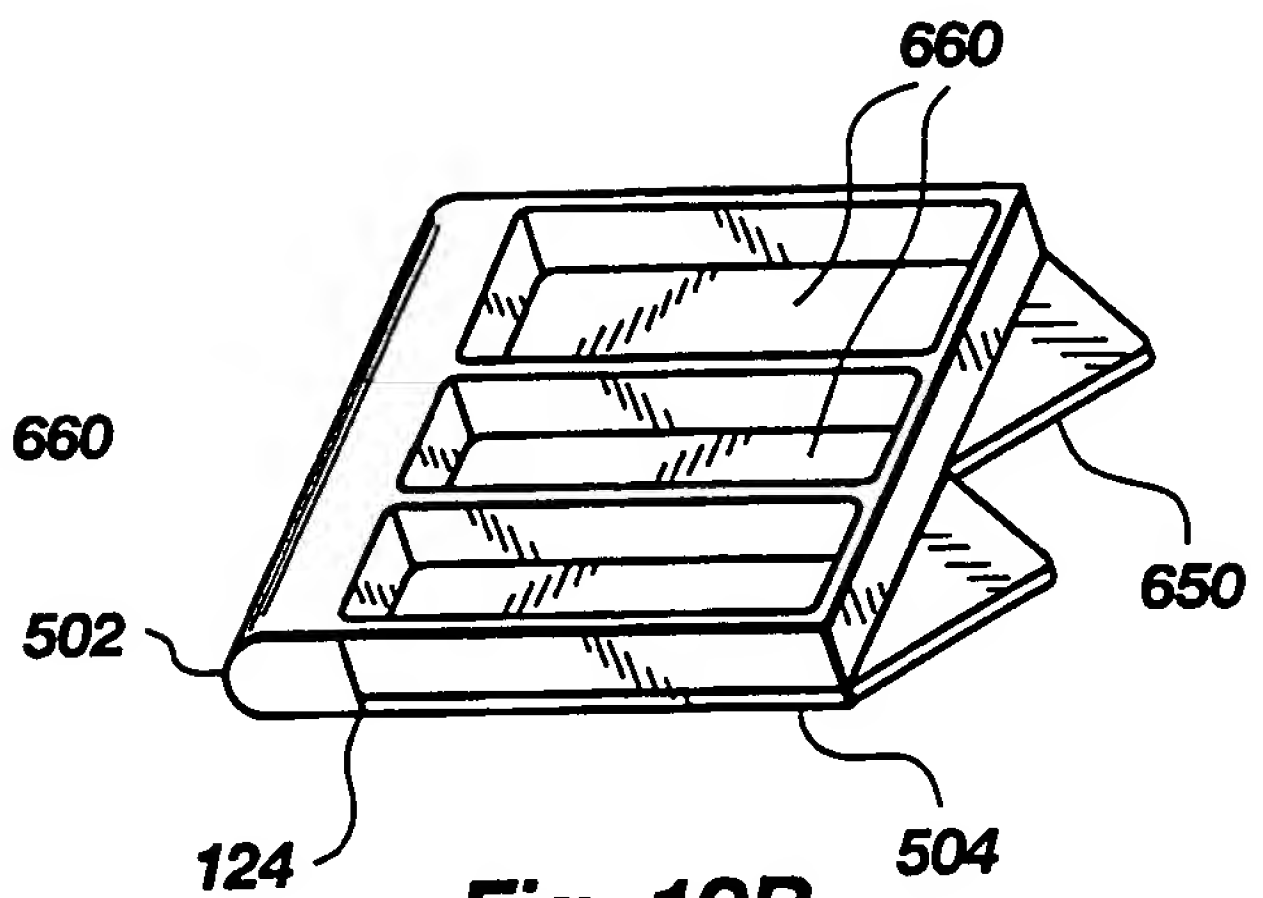


Fig. 12B

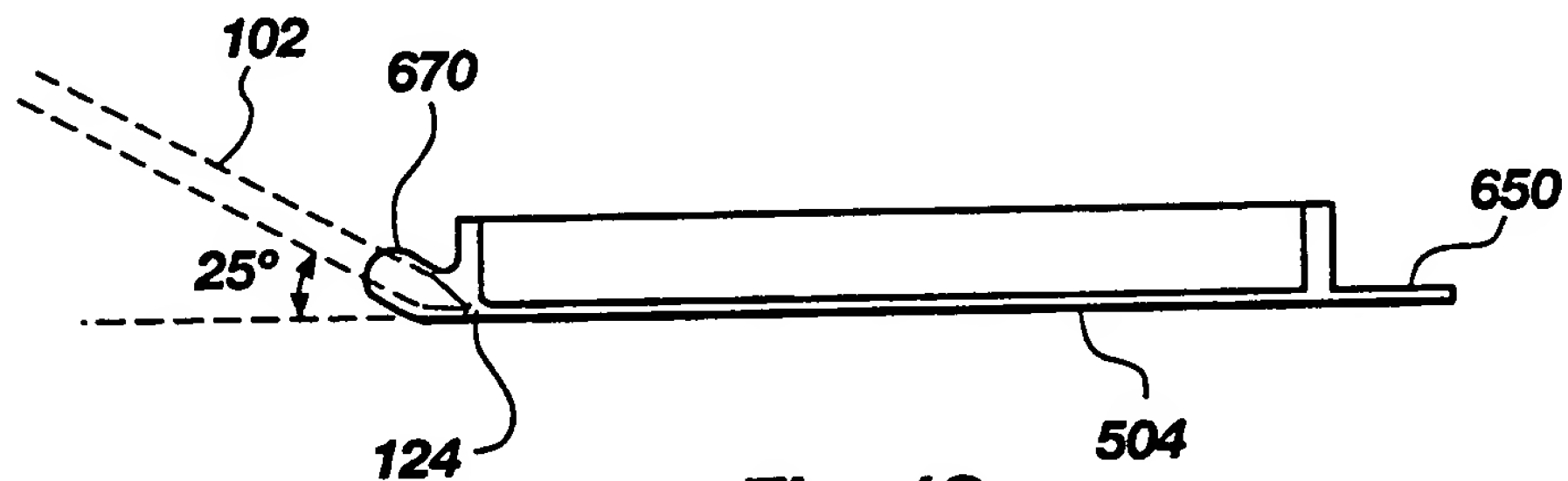


Fig. 13

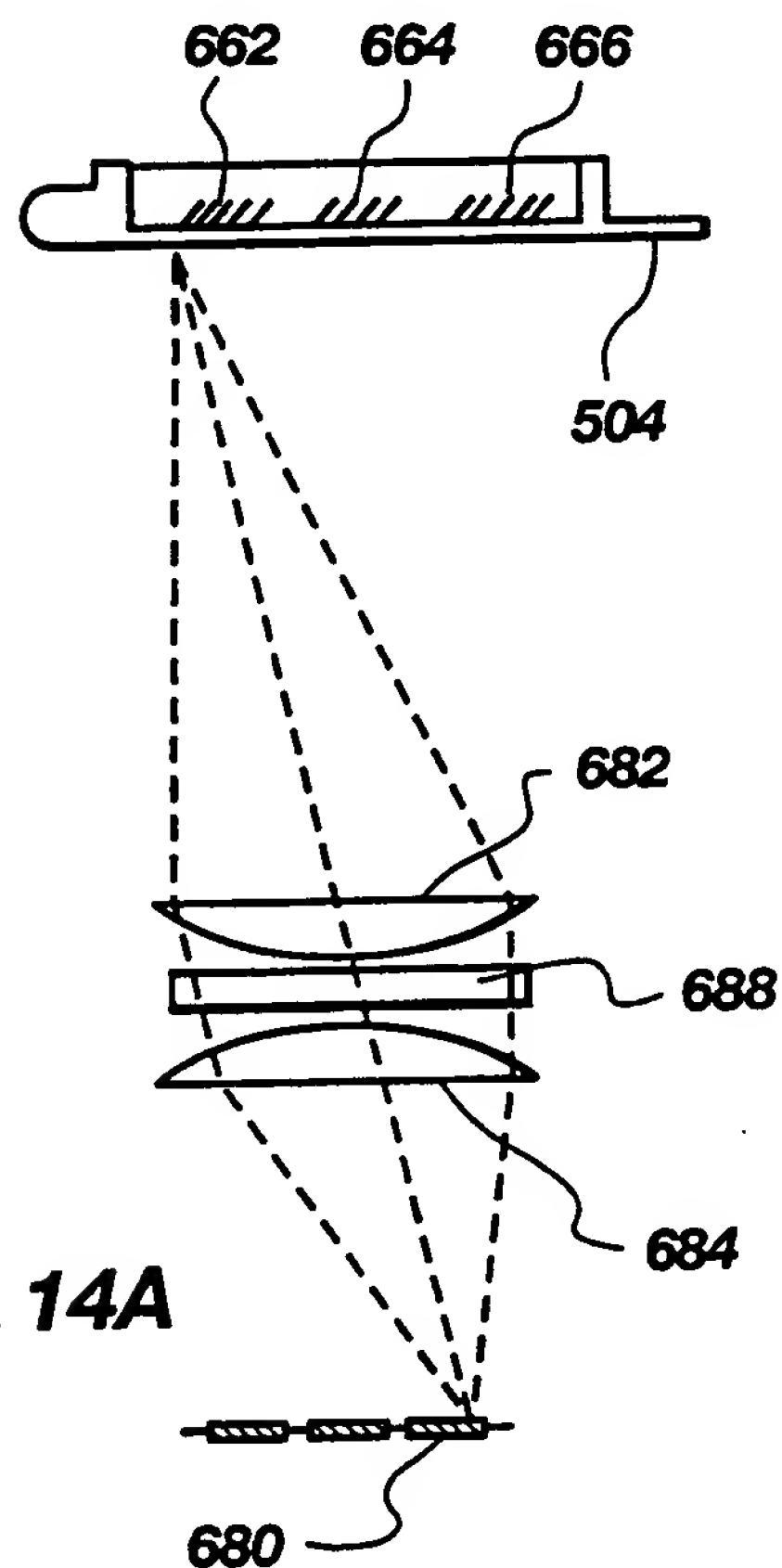


Fig. 14A

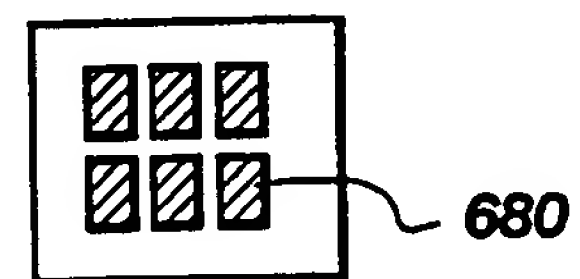
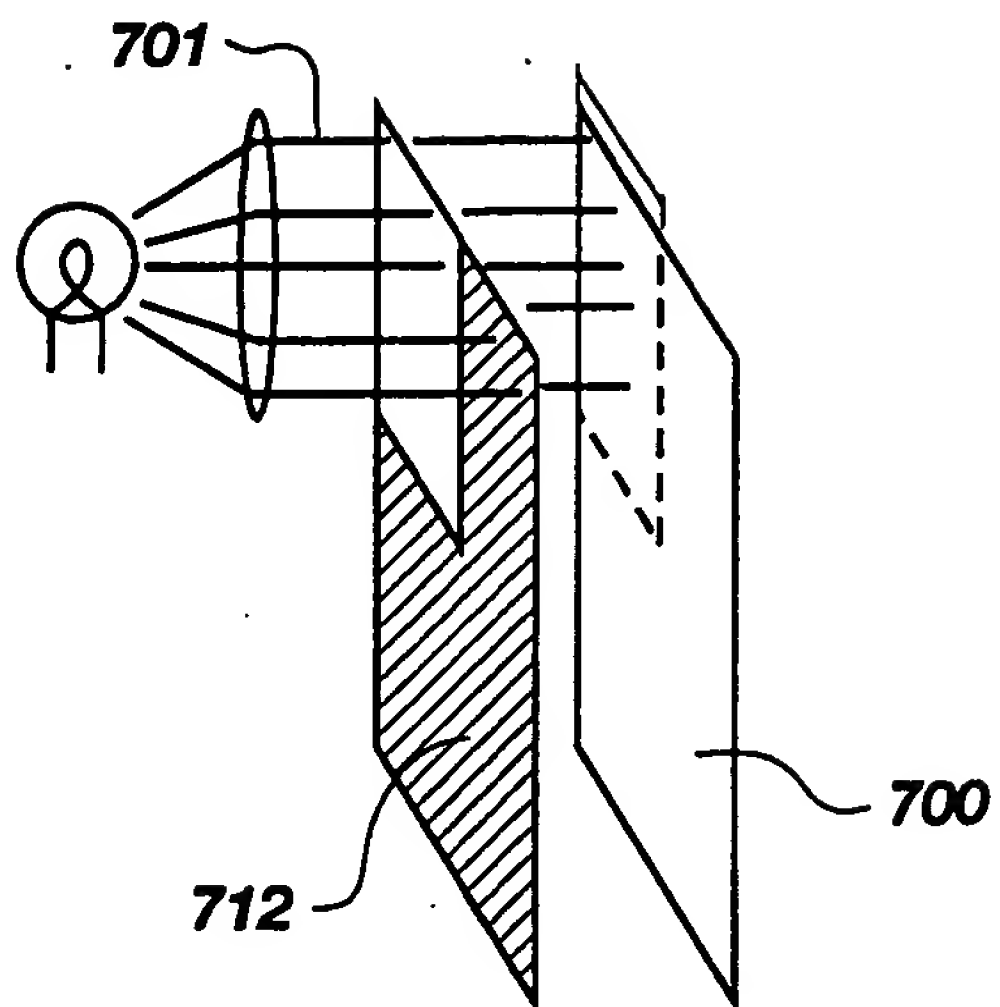
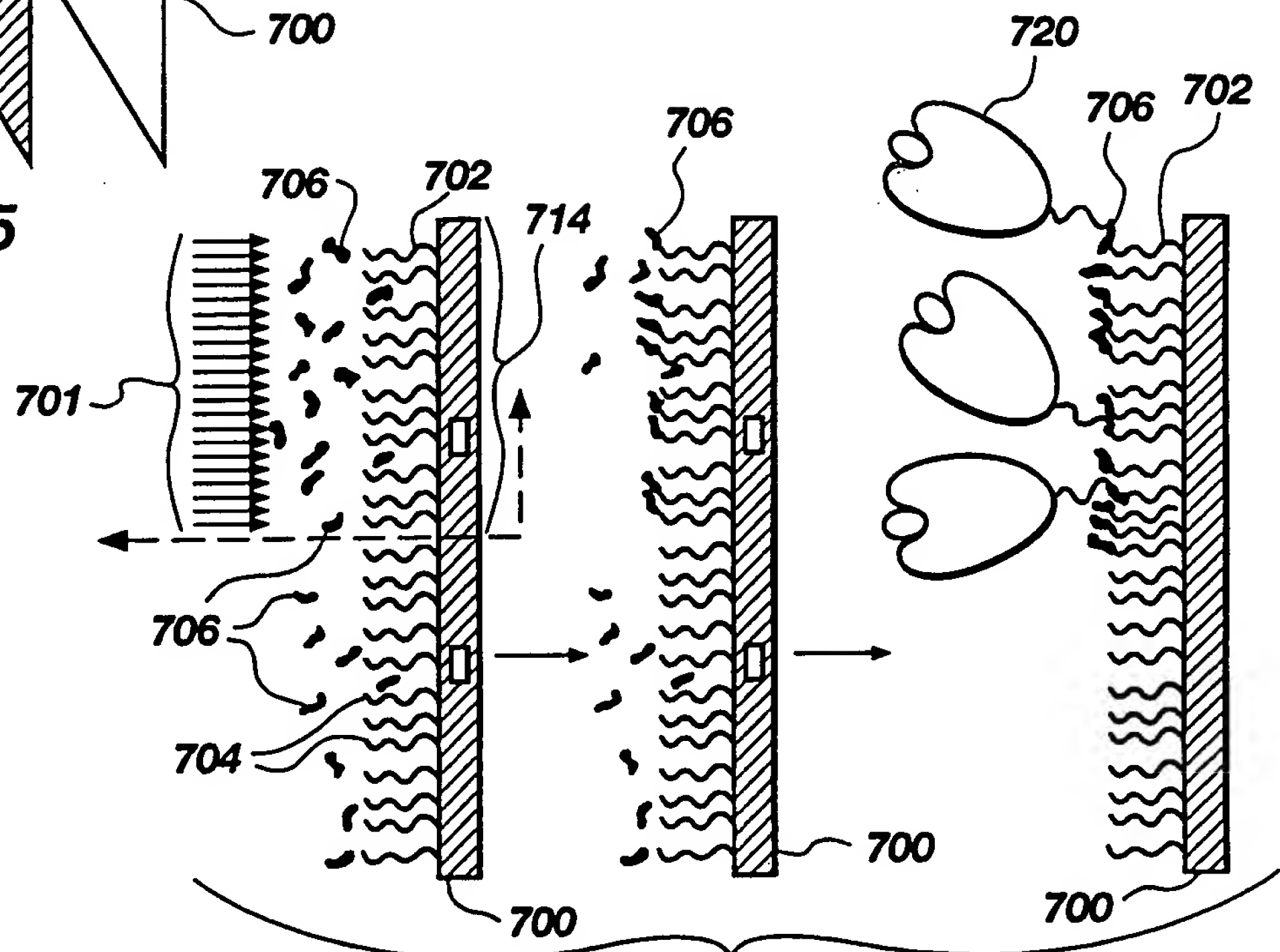
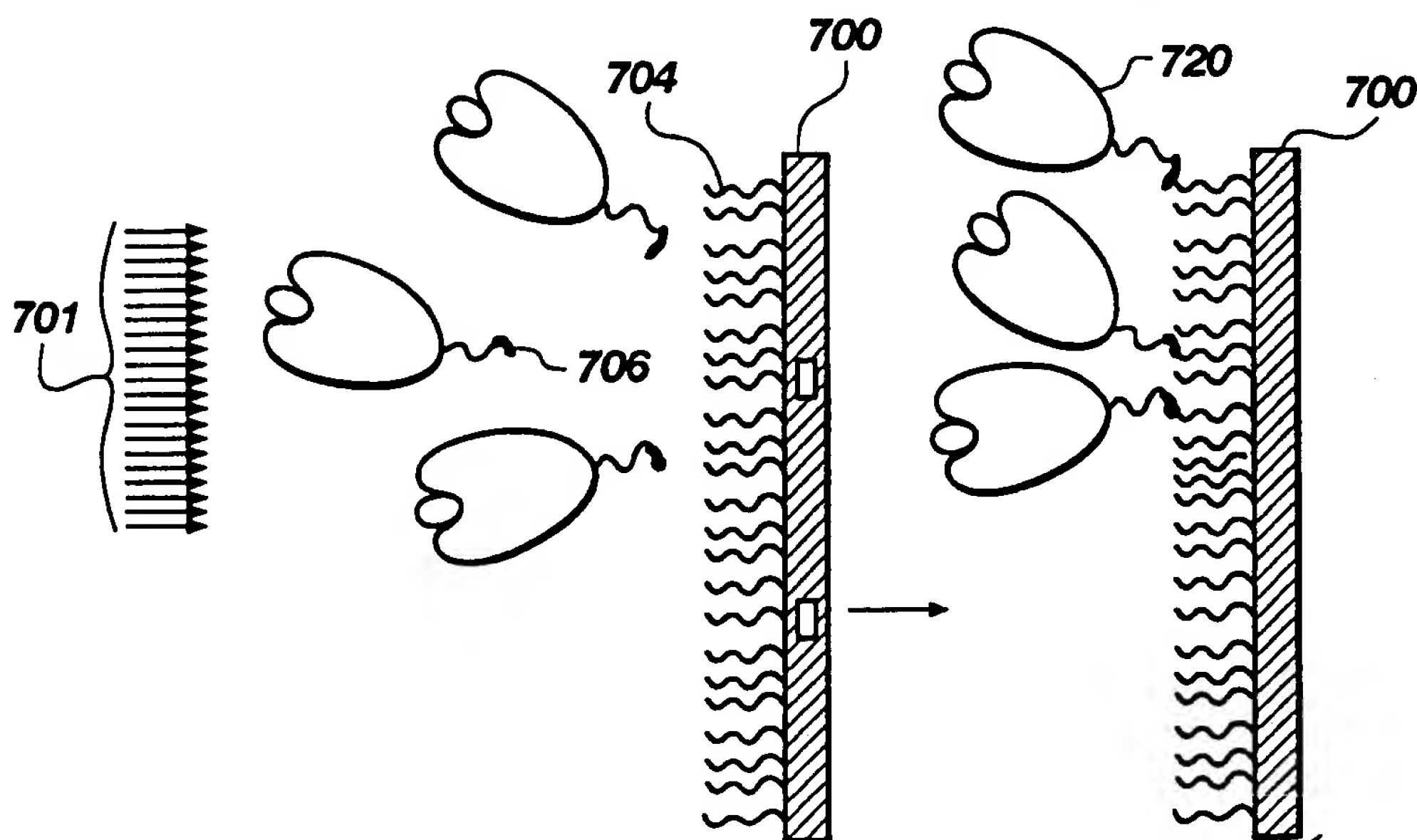


Fig. 14B



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**Fig. 15****Fig. 16****Fig. 17**

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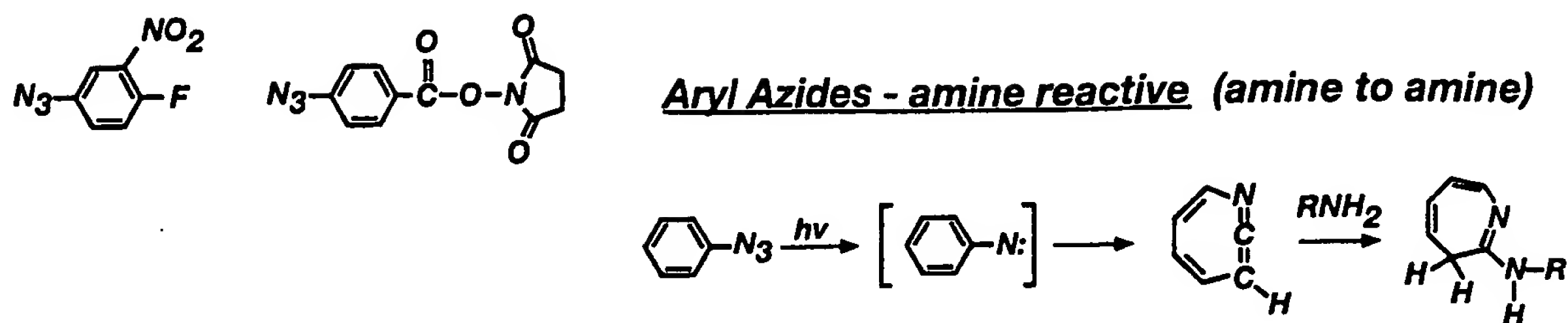


Fig. 18A

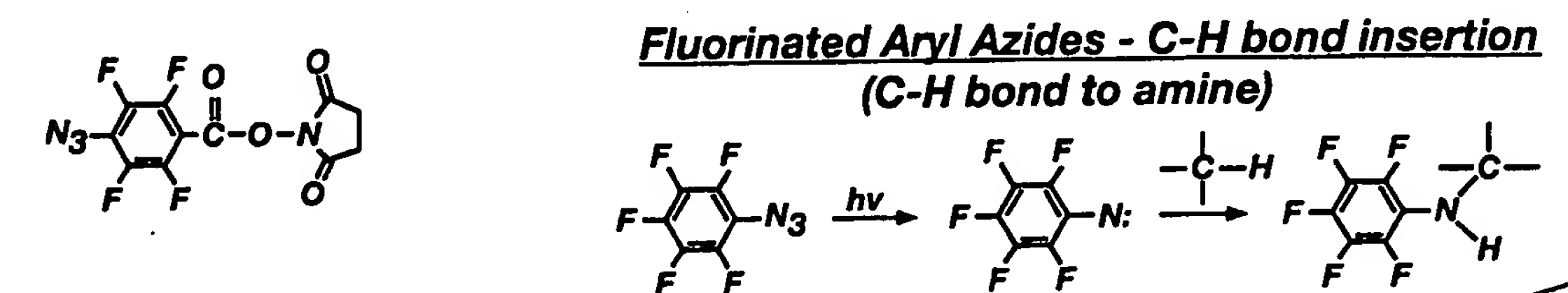


Fig. 18B

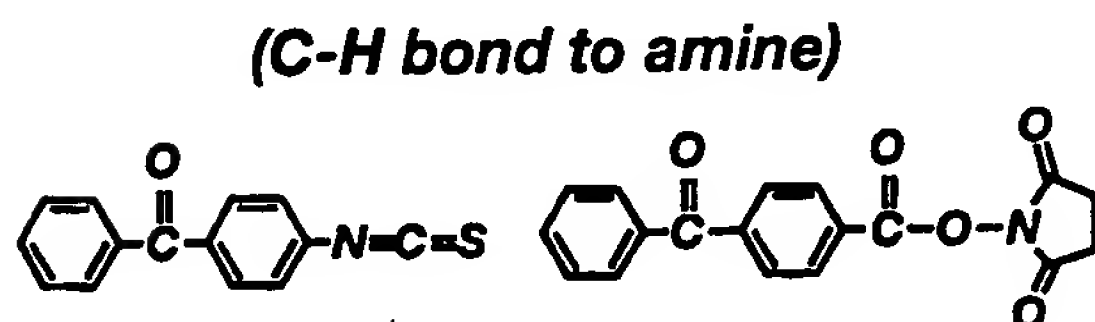
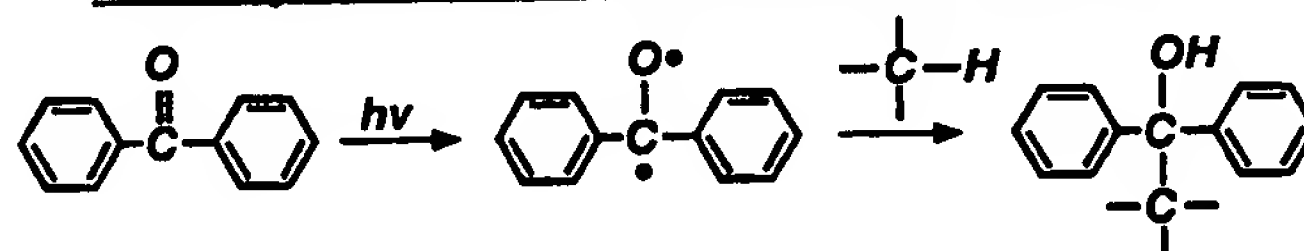
Benzophenones - C-H bond insertion

Fig. 18C

(C-H bond to thiol)

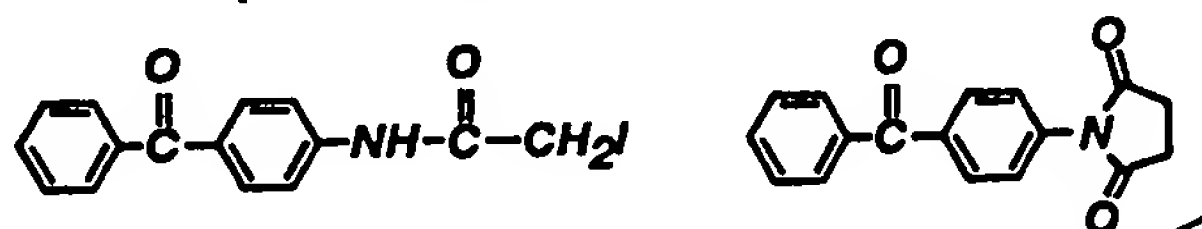


Fig. 18D